CHARACTERIZING THE EFFECTS OF PROSTAGLANDIN E_2 ON TENDON MECHANICAL PROPERTIES

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ABSTRACT

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Characterizing the Effects of Prostaglandin E_2 on Tendon Mechanical Properties
(Under the direction of Paul Weinhold)

Tendinopathy is a common clinical problem, both athletically and occupationally. It develops from repetitive mechanical loading of tendon; however the molecular mechanisms are not well understood. It is thought that prostaglandin E_2 (PGE_2) and matrix metalloproteinases (MMPs) play a role in the early stages of tendinopathy development. This research utilizes a rat tail tendon fascicle explant model to characterize the effects of PGE_2 in statically loaded and unloaded tendon. These effects were quantified through creep measurement during culture and tensile failure tests of the tissue after culture. Both specific and nonspecific MMP inhibitors were used in the system to better characterize the molecular mechanism of PGE_2's action in the tissue. We found that MMP inhibitors and static loading prevents PGE_2 from decreasing the mechanical properties of the tissue.

This work is dedicated to Jeffrey, Mom, Dad and Matt, as well as all of my other wonderful family and friends. Without your support, none of this would have been possible.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid (all-cis-5,8,11,14-eicosatetraenoic)
COX	Cyclooxygenase
DMEM	Dulbucco's Modified Eagle Medium
DMEM-H	Dulbucco's Modified Eagle Medium (High Glucose)
E	Elastic Modulus (Young's Modulus)
GAG	Glycosaminoglycan
J	Joule
MMP	Matrix Metalloproteinase
MPa	Megapascals $(kg*m/s^2)/m^2 \times 10^6$
mRNA	Messenger ribonucleic acid
NSAID	Non-Steroidal Anti-Inflammatory Drug
PGE2	Prostaglandin E2
TIMP	Tissue inhibitor of metalloproteinase
VDA	Video Dimension Analyzer
VSA	Video Strain Analysis

Chapter 1

INTRODUCTION

Tendon overuse injuries are a common medical problem both occupationally and athletically. The etiology of these injuries remains mostly uncharacterized despite their prevalence. The most frequently prescribed medications for addressing tendinopathy symptoms are nonsteroidal anti-inflammatory drugs (NSAIDs), which act to reduce pain and inflammation. However, the role of inflammation in tendinopathy has not been definitively determined. Though repetitive loading has been shown to increase inflammatory mediators in tendon tissue, biopsies from damaged tendons fail to show inflammatory cell infiltration. One such inflammatory mediator that has been implicated as a precursor to tendon overuse injury development is prostaglandin E_2 (PGE_2).

PGE2 is intimately involved in several biological processes and is found in many biological tissues, including tendon and the cervix in females. In tendon, PGE2 has long been characterized as a key factor in the development of tendinopathy over time, and in the cervix, PGE2 production is increased just before childbirth. Neither mechanism of PGE2 action is well understood; however treatments based on assumptions of PGE2 activity in these various scenarios are used regularly in the clinic. NSAIDs are prescribed in tendinopathy patients in order to decrease PGE2 and therefore hopefully lessen the

condition, and PGE2 gels are used to stimulate softening of the cervix and permit childbirth when applied to the cervix directly.

To investigate the effect of PGE2 on tendon more closely, we will employ a rat tail tendon model under static or no load conditions. The rat tail tendon model has been well-documented as an explant model for tendon *in vitro*, including static loading and stress-deprivation conditions. The static load will allow us to investigate the creep properties of tendon, which may play a role in pathology of tendon overuse injury. Since collagenase has been shown to be unable to act on tendon in uniaxial tension, the unloaded group will allow all MMPs to freely act on the tendon so their effects can be quantified biomechanically. All changes in tissue properties will be assessed via a tensile failure test immediately after culture. Decreases in mechanical properties are a clinically relevant symptom of tendinopathy, as tendons with overuse-type injuries are more likely to rupture during normal activities.

Collagenase and stromelysins have been implicated in material property changes of the cervix with PGE2 exposure. Further studies into cervical remodeling after PGE2 application has lead to a direct link between MMP-3 expression and changes in cervical tensile strength through pregnancy. No such investigation has been done to characterize PGE2's effects on MMP-3 activity in tendon, nor has the specific effect of MMP-3 in tendon been characterized. MMP-3 is a potent activator of other MMPs, including collagenases, and itself has the ability to cleave extracellular matrix proteoglycans.

Once the effects of loading and unloading tendon in the presence of PGE_2 are established, we attempted to characterize the mechanisms of PGE_2's actions. Culturing rat tail tendons with PGE2 and a broad-spectrum MMP inhibitor allowed effects due to

enzymatic degradation to be determined. Once PGE2 effects were shown to be influenced by decreasing MMP activity, the effects of MMP-3 specifically were investigated by adding a specific MMP-3 inhibitor to the culture system.

The general objective of the research presented here is to determine the effect of exogenous PGE_2 exposure on tendon biomechanical properties during unloaded and statically loaded culture. We have hypothesized that PGE_2 is acting to decrease the biomechanical properties of rat tail tendon fascicles via increased MMP activity.

Chapter 2

BACKGROUND REVIEW

2.1 Tendon Anatomy and Physiology

2.1.1 Function

Tendons are fibrous connective tissue that works to transmit loads across joints and facilitate motion. Tendons are similar to ligaments, with their main distinction being that tendons connect muscle and bone while ligaments connect bone and bone and are not subject to the same load levels as tendon. Tendons therefore have a muscular insertion site, termed the myotendinous junction, as well as a bone insertion side, or osteotendinous junction.

Tendons are passive tissues, which do not actively contract to generate forces. Instead, they transmit forces from muscles to bones to generate motion. Tendons are ideally suited for joints where space is limited, and their higher tensile strengths allow them to support large loads with minimal deformation. This process is highly efficient in that tendons forces are transmitted from muscle to bone with little energy lost to tendon stretching. They also absorb shock by damping sudden motion which limits damage to muscles and limits sudden, volatile motor stimuli [1]. A final role of the tendon is to store elastic energy, which can be converted to kinetic energy by elastic recoil resulting movement.

2.1.2 Composition

The majority of tendon is type I collagen (86%), with trace amounts of type III and V [2]. The main amino acids are glycine (33%), proline (15%), and hydroxyproline (15%). Proline and hydroxyproline give collagen fibrils their strength and therefore regions devoid of these amino acids give collagen its flexibility. Regions with the sequence Gly-Pro-Hypro have the greatest rigidity [3] and are the basis for the stability of the collagen molecule via intermolecular cross links formed by the activity of lysyl oxidase. The other major protein in tendon is elastin (2%).

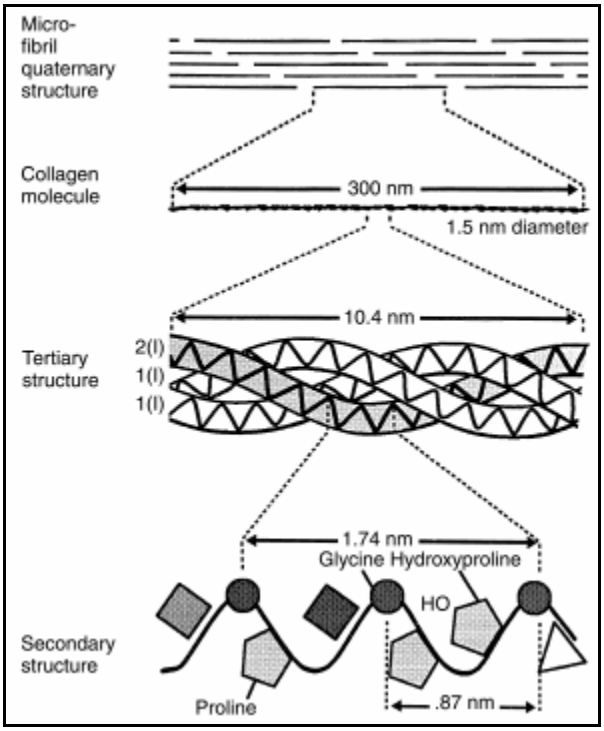

Figure 1: Structural organization of collagen into the microfibril [4]

The other major constituents of tendon tissue are proteoglycans which compose 1% to 5% of tendon dry weight. These molecules are extremely hydrophilic and bind water within the tendon. They are made up of a protein core with glycosaminoglycan

(GAG) side chains. Decorin is a small proteoglycan located on the surface of collagen fibrils, and has been proposed to connect neighboring fibrils. Decorin has also been implicated in regulating collagen fiber formation *in vivo*. It is the predominant proteoglycan in the tension-bearing portion of the tendon, whereas two types of small proteoglycans, decorin and biglycan, as well as large proteoglycans are found in the portion of tendon which undergoes compression.

The inorganic component of tendon, roughly 0.2% of the dry weight, include copper, manganese, magnesium, cadmium, cobalt, zinc, nickel, lithium, lead, fluoride, phosphor and silicon. Their roles are various, some yet unknown, but some well defined ones include copper's role in cross-linking, manganese's role in molecule synthesis and calcium's role in signaling [5].

Finally, the cells of tendon are called tenocytes, which are specialized fibroblasts that maintain the collagen structure of the tissue. About 95% of the cells found in tendon are tenocytes; however there are also chondrocytes at insertion points of the tendon, synovial cells in the tendon sheath, vascular cells and inflammatory cells during certain pathologic conditions.

2.1.3 Structure and Organization

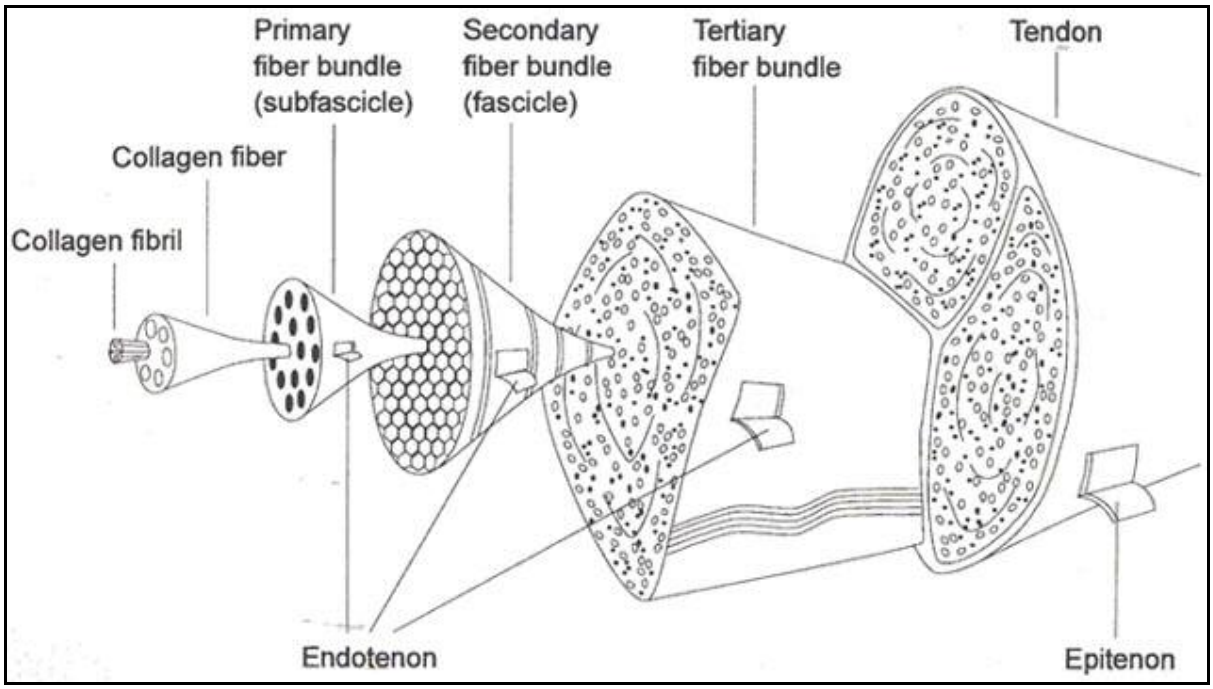

Figure 2: Schematic of tendon microarchitecture[6]

The collagen fibrils are long rod shaped structures composed of numerous triple-helix polypeptide chains. The chains are surrounded by a thin layer of proteoglycans (PGs) and glycosaminioglycans (GAGs). The molecule is roughly 300nm long and 1 – 2nm wide. Each molecule overlaps its neighbors by 67nm or roughly 25% and is often referred to as the quarter-staggered array. This 67nm region is comprised of an overlap region of 40% and a hole region of 60% [3]. These fibrils are surrounded by more PGs and GAGs that are different from those surrounding the alpha chains [1].

2.1.4 Mechanical Properties

Tendons are capable of supporting extremely large loads with minimal strain. Tendons are passive tissues and cannot actively contract to generate forces. Thus, the

ability of tendons to support large loads is dependent upon the properties of the extracellular matrix molecules: collagen, proteoglycan and water.

2.1.4.1 Failure Properties

Generally, mechanical properties of tendons are evaluated *in vitro* by uniaxial tension tests. A typical failure curve from this type of test is shown in Figure 3. The shape of the curve is due to the viscoelastic properties of tendon. At low strains, less stiff elastic fibers are supporting the majority of the load and the crimped pattern is gradually straightened. This requires minimal force to see strains of 0-2% develop in the tissue and is known as the toe region [7]. . After the crimp is straightened, the tendon is much stiffer. This linear region of the stress-strain curve ranges from 1.5 – 4% strain and is elastic so the strain is fully recoverable [7, 8]. Past 4% strain is the failure region where non-recoverable damage to individual fibrils occurs as they begin to shear past each other and cross links break in an unpredictable fashion [4, 7, 9]. This continues until enough fibrils and cross-links are damaged to cause macroscopic failure, which commences around 8-10% stain [7, 9].

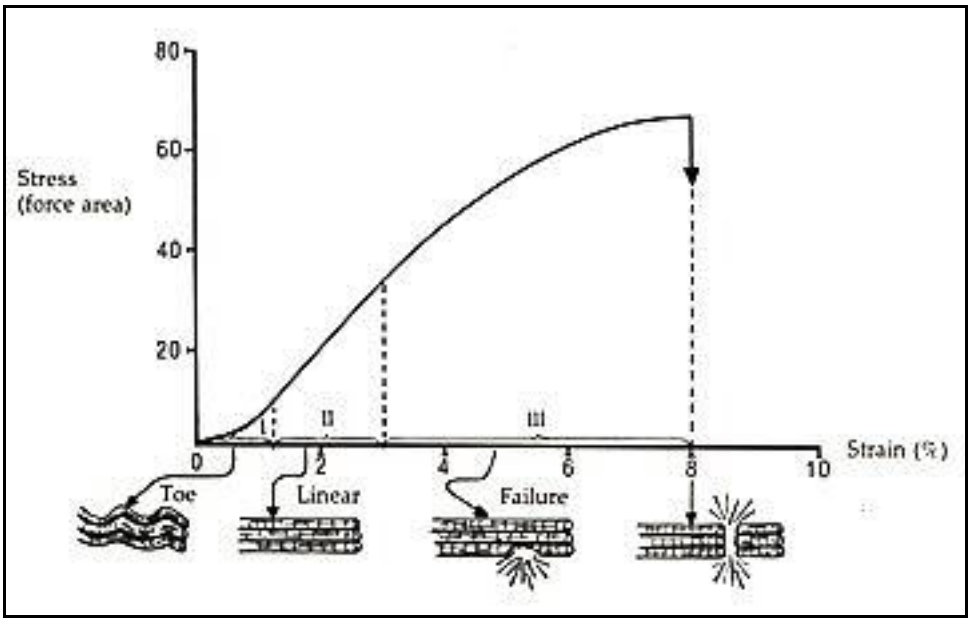

Figure 3: Typical Stress-Strain Curve for Tendon

2.1.4.2 Strain Rate Dependence

The viscoelastic nature of tendon results in a load-rate dependent stiffness of the tissue. As tendons are strained, fluid in the collagen fibers flows out of the tendon. Thus, at high strains a majority of the applied load is bore by the collagen. However, if the tendon is stretched rapidly, the fluid is unable to flow out completely resulting in increased stiffness [10]. This effect is illustrated in Figure 4.

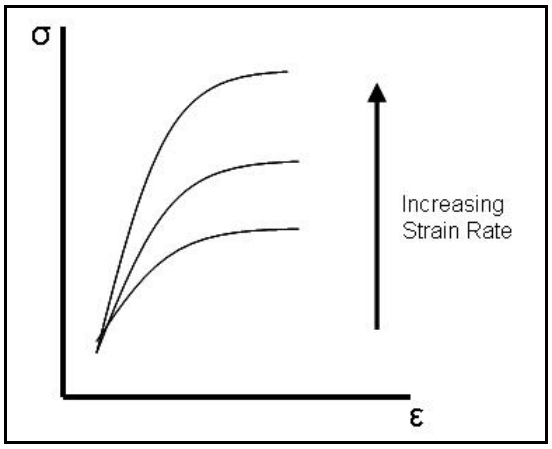

Figure 4: Strain Rate Dependent Stress-Strain Curves for Viscoelastic Materials

2.1.4.3 Hysteresis

Another viscoelastic behavior of tendon is that of preconditioning, a phenomenon where the first few cycles of load result in greater work being done to stretch the tendon than is recovered when the tendon is allowed to relax. Hysteresis is the difference in energy imparted to and returned from a material, where this energy is lost from the system as heat [4]. The hysteresis effect on stress is shown in Figure 5.

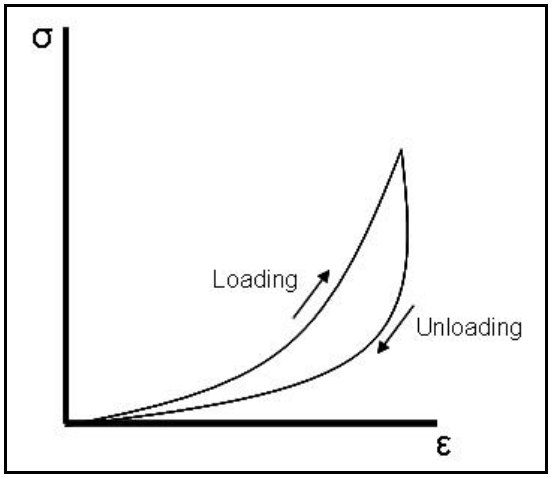

Figure 5: Hysteresis Loop of Tendon

2.1.4.4

Tendon will undergo creep with application of a constant applied load, as shown in Figure 6. Physiologically, this is beneficial, as during muscle contraction the tendon-muscle unit length remains constant. As the tendon gradually lengthens, the muscle can relax. This reduces muscle fatigue, and increases performance during contraction [4]. However, Goldstein *et al.* have shown that creep may be an important factor in cumulative tendon disorders [11]. Prolonged creep can lead to tensile failure at much lower stress levels than a dynamic failure test, and in tendon this time is depended upon the initial strain amount placed on the tendon [12].

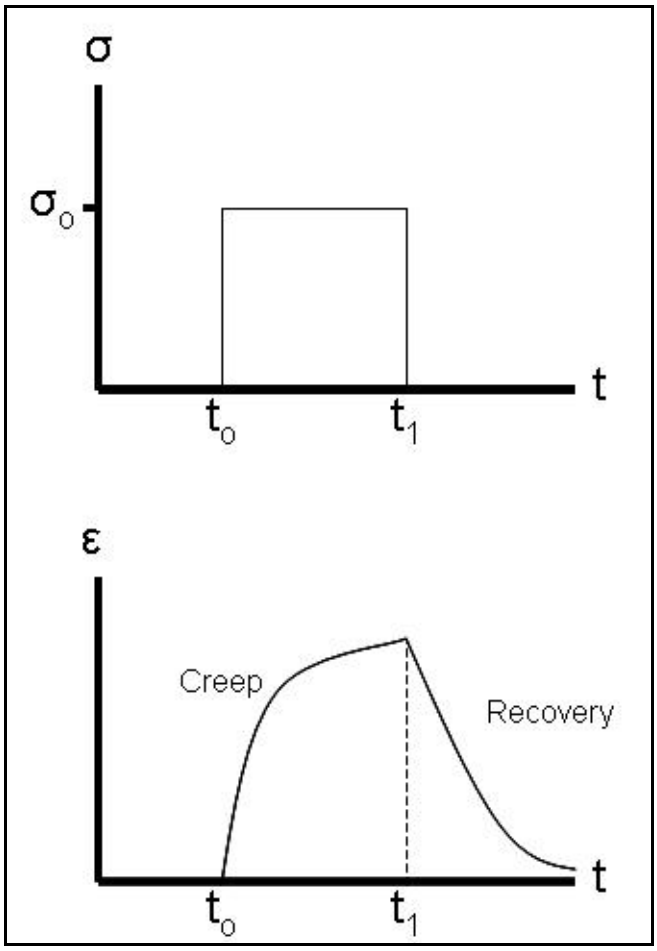

Figure 6: Creep and Recovery

2.2 Tendon Loading

2.2.1 Disuse

Immobilization has serious negative effects on tendon. Examining immobilized joints of both animals and humans has shown reductions in GAG content and increased collagen type III, resulting in decreased tensile strength, elastic stiffness, total weight and energy to failure [1, 13-17]. Also, MMP-1 expression is significantly upregulated with stress deprivation in animals [18]. Moreover, the tensile loading tendon *in vitro* has shown to have a protective effect by inhibiting the ability of collagenase to cleave collagen molecules in loaded tendon, as well as protecting collagen bundles from inflammatory cell-induced deterioration [19].

2.2.2 Normal Use

Long-term physical activity improves the tensile mechanical properties of tendons [16]. Results from normal activity result in the opposite effects seen with tendon disuse. Training induced changes include increased GAG content, decreased collagen cross-linking and increased collagen fiber alignment [16]. These changes have been shown to increase load at failure in rabbits [20].

Using microdialysis, *in vivo* measurements of human Achilles tendon response to exercise has been measured. Inflammatory mediators prostaglandin E_2 and thromboxane B_2 were increased during the exercise, and levels remained raised after a 60 minute recovery period [21].

2.2.3 Overuse

Although appropriate training or exercise produces positive effects on tendons, excessive loading during vigorous exercise or occupational activity with a high frequency and/or long duration may induce tendon degeneration. In rats, extensive training over a 4 week period decreased the elastic modulus of the supraspinatus tendon, and the stress level at failure [22]

Overuse injuries in tendon are thought to arise from repetitive tensile loading that results in subfailure microinjuries, and the cumulative effect results in deterioration [23]. The molecular mechanisms of this degeneration are not well understood, though fibril damage has been linked to increases in collagenase mRNA and protein levels [24]. The failure of an individual fibril results in a mechanobiological understimulation of the tenocytes associated with that fibril, resulting in the effects seen in stress deprived tendon including increased collagenase. This may increase damage to the extracellular matrix and increase the risk for further damage as load is repetitively applied [25].

2.3 Tendinopathy

Tendinopathy is the general term used to describe disorders of the tendon, such as tendonitis, tendinosis and paratendinitis. The pathogenesis of tendinopathy is difficult to study in patients, as tendon biopsies are only obtained after the condition has become chronic. However, it is assumed that tendinopathy develops with repetitive mechanical loading and some molecular mechanisms that are not well characterized. Load inducible cellular responses that likely play a key role in driving tendon degeneration are cytokines,

inflammatory mediators and MMPs [26, 27]. There is some discrepancy as to the role of inflammation versus degeneration in development of the disease.

2.3.1 Degeneration

MMPs are the primary proteins used to remodel the collagen matrix, and thus their potential role in tendinopathy is direct and obvious. They can be converted from an inactive proenzyme to active enzymatic form by specific MMPs, and thus a positive feedback loop can be activated that leads to excessive degeneration. Injection studies of collagenase have shown tendinosis like degeneration, similar to that observed during tendon injury, thus implicating the collagenase MMPs as potential tendinopathy contributors [28, 29].

2.3.2 Inflammation

Cytokines and inflammatory mediators, such as IL-1β and PGE_2, act indirectly through a host of cascades that can lead to tendinopathy. Injection models of both cytokines and inflammatory mediators have shown tendinosis type degeneration [28, 30]. Though their exact mechanism in tendinosis is unclear, and the exact role has been questioned [31], inflammatory molecules have long been implicated as key to early development of tendinopathy.

2.4 Matrix Metalloproteinases

Degradation of extracellular matrix (ECM) proteins is initiated by zinc and calcium dependent endopeptidases called MMPs. These enzymes are secreted from cells in the pro form, and must be activated before degrading ECM components [32]. The mechanism of proMMP activation is poorly understood *in vivo*.

There are 23 members of the MMP family, and they are divided into groups based on substrate. The four main MMP classes are collagenases, gelatinases, stromelysins and membrane type MMPs.

2.4.1 Collagenase

MMPs with collagenase activity include MMP-1, 2, 8, 13 and 14 [33, 34]. The importance of collagenase in development of degenerative tendinopathy has not yet been fully investigated.

MMP-1 mRNA has been shown to be increased *in vitro*, as tenocytes are exposed to fluid shear stresses, and *in vivo*, in ruptured supraspinatus tendon [35, 36]. MMP-1 increases are also seen in stress deprived tendons in culture, and can be eliminated by exposing the tenocytes to a cyclic tensile load [18]. This same MMP-1 mRNA inhibition is seen in rat tail tendons cultured in static loading conditions [37]. Thus, the type of loading (shear versus cyclic and static tensile loads) seems to be an important factor in MMP-1 expression in tendon.

MMP-2 has been shown to degrade ECM at the tendon edges in an acute tear animal model [38].

2.4.2 Stromelysin

The stromelysins consists of MMP-3, 10, and 11. These MMPs degrade proteoglycans, fibronectin, casein, and collagen types III, IV and V. They are also potent activators of other MMPs (such as MMP-1, 3, 7, 8, 9 and 13), and thus have the potential to be an important regulator of tendon remodeling.

A decrease in MMP-3 mRNA is associated with the changes seen during tendinopathy, along with a decrease in the mRNA of a number of TIMPs [39]. This may indicate a potential role for MMP-3 in non-pathologic tissue maintenance. Investigation into the timing for MMP-3 activity of tendon could potentially uncover more information about the onset of tendinopathy.

In vitro studies have shown rabbit tendon cells increase expression of MMP-3 with exposure to shear stress [35]. Shear stress on tenocytes may contribute to tendinopathy by way of action of MMPs and COX-2 [35, 40]. It has been suggested that certain individuals may have an increased susceptibility to tendon disorders, due, in part, to their tenocyte's altered production of MMP-3 in combination with a decrease in TIMP production [40].

2.4.3 Tissue inhibitors of metalloproteinases

MMP activity is inhibited by tissue inhibitors of metalloproteases (TIMPs). In normal tendon, MMPs and TIMPs are balanced to regulate tissue remodeling. However, tissue samples from patients exhibiting tendinopathy symptoms show an increase in MMP mRNA as well as a decrease in TIMP mRNA [41, 42]. Table 1 indicates some common MMPs and TIMPs found in tendon.

TIMP-1 is not normally present, but its expression is increased at the site of an acute tear. Therefore, it may be responsible for inhibiting excessive MMP-2 degradation of tendon [38]. TIMP-1 is also thought to be an important regulator of MMP-1, and a significant decrease in TIMP-1 activity is seen in tendinopathic tendon [43].

Table 1: Main roles of some MMPs and TIMPs in tendon [40]	
MMP/TIMP	**Main Roles**
MMP-1	Degrades type I collagen Up-regulated in acute tendon tears Up-regulated in tendinopathy Up-regulated in response to shear stress Up-regulated during stress deprivation Down-regulated in response to cyclic strain and static tensile load
MMP-2	Up-regulated in tendinopathy May be up-regulated or down-regulated in complete tendon tears Inhibits TIMP-1 and TIMP-2 in response to exercise
MMP-3	Plays a major role in maintenance and remodeling of normal tendon Down-regulated in tendinopathy and complete tendon tears Up-regulated in response to shear force
MMP-13	Up-regulated in complete tendon tears
MMP-9	Up-regulated following exercise
TIMP-1	Down-regulated in tendinopathy Up-regulated transiently following an acute tendon tear Inhibits excessive degeneration by MMP-2
TIMP-2	Down-regulated in tendinopathy and complete tendon tears

2.5 Arachidonic Acid

Arachidonic acid is an omega-6 fatty acid with a 20-carbon chain and 4 *cis* double bonds. It is present in the phospholipid bilayer of cells, and is essential to membrane fluidity at physiologic temperatures. Arachidonic acid is the initial molecule in a cascade that ends in prostaglandin E_2, along with a variety of other prostanoids.

The arachidonic acid cascade can be started by an inflammatory response. Initially, phospholipase A2 releases free arachidonic acid from the lipid bilayer. Once freed, arachidonic acid can undergo enzymatic conversion to prostaglandins, thromboxanes, prostacyclins and leukotrienes. However, unaltered arachidonic acid has been suggested to have several physiologic roles.

At rest, the concentration of free arachidonic acid is low. When exogenous arachidonic acid is introduced it has been shown to be absorbed and metabolized by the cells [44]. Whether artificially placed or naturally occurring, free arachidonic acid in the cytoplasm has three fates: storage in the cell membrane, diffusion outside of the cell, or metabolism.

When arachidonic acid moves outside the cell it may be involved in a range of functions. The leukocyte NADPH oxidase can be activated to reduce molecular oxygen to superoxide by micromolar concentrations of arachidonic acid [45, 46]. Polyunsaturated fatty acids in general play a role in many ion channels. For example, mechano-sensitive K^+ channels, Ca^{2+} channels, gap junctions, and a dopamine transporter have all been reported to show an action of polyunsaturated fatty acids [47-50]. More specifically, a member of the K^+ channel is called the TWIK-related arachidonic acid-stimulated K^+ channel or TRAAK [51]. Arachidonic acid has been shown to inhibit cell proliferation and initiate apoptosis [52]. Arachidonic acid-induced apoptosis may be the mechanism behind the protective effect of NSAID usage on the incidence of colon carcinoma, due to arachidonic acid accumulation in patients taking these drugs [53].

Arachidonic acid's many broad physiologic effects can be attributed to its simple structure and many close chemical analogues. Any specificity in its actions may be simply due to its relative specificity of release from the membrane in certain situations when compared to other fatty acids. The concentration of arachidonic acid required to elicit the above biologic responses is usually on the micromolar level, and when compared with the nano- or picomolar levels of the prostaglandins or leukotrienes required to evoke cellular responses, arachidonic acid is clearly less potent.

Arachidonic acid metabolism is the more interesting fate of the freed molecule for the work presented here, the pathway is illustrated in Figure 7. The arachidonic cascade is driven by three main enzymes: cytochrome P450, lipoxygenase and cyclooxygenase. Cytochrome P450 converts arachidonic acid to epoxyeicosatrieonic fatty acids (EpETrEs). Lipoxygenase and cyclooxygenase convert arachidonic acid to eicosanoids, including leukotrienes, prostanoids and thromboxanes.

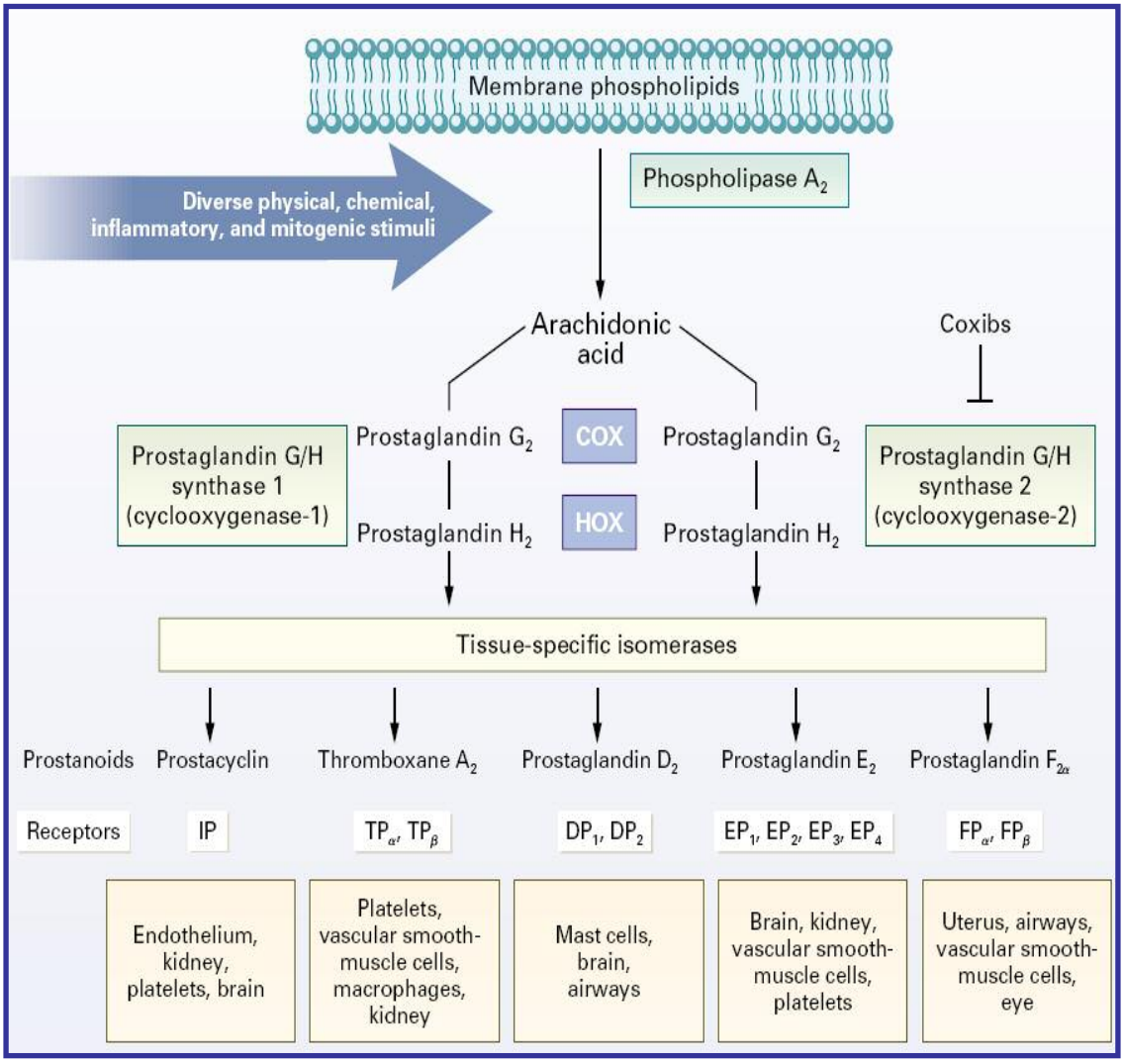

Figure 7: Arachidonic Acid Pathway [54]

2.5.1 Lipoxygenase

Lipoxygenases are an entire family of enzymes that act to oxygenate arachidonic acid. The lipoxygenase pathway is the metabolic transformations that arachidonic acid undergoes during conversion by these enzymes.

The three main isoforms of lipoxygenase are 5-Lipoxygenase (5-LO), 12-Lipoxygenase (12-LO), and 15-Lipoyxgenase (15-LO) forming 5-, 12-, and 15-hydroperoxy-eicosatraenoic acid (HPETE), respectively. The lipoxygenase pathway refers to the metabolic transformation that the HPETEs undergo. HPETEs are converted to lipoxins, 12-HETEs and leukotrienes of the 4 series.

The 5-LO pathway produces leukotrienes, which are eicosanoid lipid mediators that act principally on G protein coupled receptors. They are primarily involved in allergic reactions and inflammatory conditions, such as rheumatoid arthritis, psoriasis and asthma. Leukotriene B4 is a chemoattractant for neutrophils, macrophages and other inflammatory cells and induces adhesion of these cells to the vascular endothelium [55]. Leukotrienes C_4, D_4 and E_4 increase vascular permeability and contract smooth-muscle cells [55]. 5-LO requires an activator protein from the nuclear membrane known as the 5-lipoxygenase activating protein (FLAP).

Little is known about the 12-LO pathway, it was originally isolated in human and animal platelets and has since been isolated in porcine leukocytes and other tissues [56-58]. Its biologic functions have been shown to range from cardiovascular to neurological applications, but it does not produce potent biological mediators such as prostaglandins, thromboxanes and leukotrienes [59-61]. Its main role is regulating cellular

concentrations through apoptosis or proliferation in inflammatory and normal tissue maintenance.

HPETEs are converted to lipoxins A4 and B4 via the 15-LO pathway. Lipoxins are eicosanoids that appear at the end of an inflammatory response and are considered the "braking signals" of inflammation. The lipoxins have been shown to have vasoactive properties at nanomolar concentrations; stimulating vasodilatory responses in some tissues and vasoconstricting responses in others [62]. They also have potential roles as immunoregulators and have been shown to inhibit cell migration and proliferation [62, 63].

2.5.2 Cyclooxygenase

Cyclooxygenase (COX), also known as prostaglandin H synthase, is the enzyme required for conversion of arachidonic acid to prostaglandins. It catalyzes the production of prostaglandin G_2 (PGG_2) from arachidonic acid, and then peroxidase reduces PGG_2 to PGH_2. Then several cell-dependent synthases convert PGH_2 into the various prostanoids. There are three isoforms of the COX enzyme, COX-1, COX-2 and, recently discovered, COX-3 which is a COX-1 variant.

It has been shown that COX-1 is constitutively expressed in many cells, whereas COX-2 is induced by certain stimuli, such as inflammation, pain, fever and cancer. COX-3 appears to be selectively inhibited by acetaminophen over COX-1 and -2, and this inhibition has been presented as a possible mechanism in pain and fever reduction [64]. Determining the specific functions of COX is key, as these enzymes are the main targets for non-steroidal anti-inflammatory drugs (NSAIDs).

Both COX-1 and COX-2 are integral membrane proteins in the endoplasmic reticulum (ER) and perinuclear envelope, with COX-1 more enriched in the ER, and COX-2 in the perinuclear envelope [65, 66].

The role of COX in physiologic and pathologic conditions has been thoroughly investigated with the use of mice deficient in COX-1 or COX-2. COX-2 deficient mice show a milder inflammatory response to rheumatoid arthritis, carageenan-induced edema and subdermal air pouch then COX-1 deficient or wild-type mice [67, 68]. However, arachidonic acid-induced edema is milder in COX-1 deficient mice than in COX-2 deficient mice, indicating both isoforms play a role in some type of inflammation [69, 70].

These mouse models have also been used to study the role of COX in female reproduction. COX-1 deficient mice have significantly prolonged gestation periods [71]. COX-2 knockout mice have many female reproductive problems with ovulation, fertilization and implantation [72].

After conversion of arachidonic acid to PGH_2 by COX and peroxidase, specific synthases convert PGH_2 to thromboxanes, prostacyclins and prostaglandins D, E, and F. Thromboxane is produced by platelets via thromboxane-A synthase, and acts as a vasoconstrictor and plays a role in clot formation. Thromboxane is in homeostatic balance with prostacyclins, which is produced in endothelial cells via prostacyclin synthase. Prostacyclin acts as a vasodilator and prevents platelet formation and clotting. Prostaglandins are produced in most cell types via prostaglandin synthase. Their main action is in inflammation mediation; however they also regulate calcium movement, cell growth and hormone regulation.

2.6 Prostaglandin E_2

PGE2 is an inflammatory mediator that has vasodilatory effects at locations of inflammation. It also acts to increase cAMP levels in neutrophils and macrophages and inhibits their functional response to other inflammatory stimuli. With respect to tendon, PGE2 has been shown to decrease collagen type I production as well as increase MMP-1 and 3 expression and synthesis [73]. PGE2 is also utilized clinically to induce cervical softening during the onset of labor. The role of collagenase in these changes has not been definitively shown [74-76], but MMP-3 increases have been identified as a primary cause of cervical tensile strength changes with PGE2 exposure [77].

PGE2 acts through EP receptors. These are G-protein-coupled receptors which are classified into four subtypes: EP_1, EP_2, EP_3 and EP_4 [78]. Once activated, each receptor initiates a unique signal transduction mechanism. EP_1 stimulates intracellular calcium, EP_2 and EP_4 activate increases of intracellular cAMP levels, and EP_3 has been implicated in multiple signal pathways [79]. In fibroblasts, PGE2 has been shown to act through upregulation of specific EP-receptors to achieve various effects. Lung fibroblasts, which activate EP_2 receptors to reduce collagen type I expression, show decreased ability to suppress collagen synthesis with PGE2 exposure in EP_2-deficient mice [80]. It has been proposed that the specific upregulation of EP_4 receptors in tendon fibroblasts may trigger inflammatory signaling pathways leading to tendinopathy [81]. In the cervix, EP4 has been implicated as the PGE2 receptor involved in producing the tissue changes at term [82].

2.6.1 PGE2 effects in tendon

PGE2 has been identified in tendon as a central mediator in inflammation and pain [83]. Models of tendinopathy have provided evidence that PGE2 production may be an important step on tendinopathy development [30, 84, 85]. Many studies, *in vivo* and *in vitro*, have been conducted in order to better understand the cellular and molecular mechanisms of tendinopathy. It has been shown that PGE2 is released by human tendon fibroblasts after cyclic mechanical stretching *in vitro* [85, 86], and PGE2 has also been quantified in the peritendinous space after exercise in an *in vivo* study [21].

PGE2 has been shown to decrease human patellar tendon fibroblast proliferation and collagen production when cultured *in vitro* [73]. Furthermore, *in vivo* studies in rabbits have shown that PGE2 injection into the patellar tendon leads to disorganization of the collagen matrix and decreased diameter of collagen fibrils [30]. Interestingly, however, there was an absence of inflammatory cells in the tendon after one week, and tendon mechanical properties were not assessed. The observed changes in these tendons has been proposed to be due to an up regulation of collagenase by PGE2 [30]. PGE2 is thought to mediate MMP-3 production and activation in fibroblasts [81, 87].

Previous studies in our laboratory have shown a trend for exogenous PGE2 exposure to increase accumulated strains during cyclical loading in an avian tendon explant model. This is consistent with past results from cultured fibroblast and human micro-dialysis studies which both showed increased PGE2 after loading [21, 84, 88, 89]. The studies in our lab resulted in load-induced PGE2 levels similar to the human tissue levels seen in the micro-dialysis studies [27]. The established role of PGE2 as an inflammatory mediator coupled with its increase with loading have prompted the consideration of excessive PGE2 production to play a role in the pathology of overuse

tendinopathy [90]. Furthermore, application of COX inhibitors in our laboratory's model was shown to significantly decrease accumulated strains with cyclical loading thus providing additional evidence of a role of PGE2 in facilitating the lengthening of dense connective tissues. Evaluation of mechanical properties from failure tests from this study revealed the midsubstance stiffness of the tissue to be decreased and the strains at ultimate load to be increased with PGE2 treatment. However, no differences in strength were observed with PGE2 treatment, which may provide evidence that PGE2 acts through a pathway other than collagenase activation.

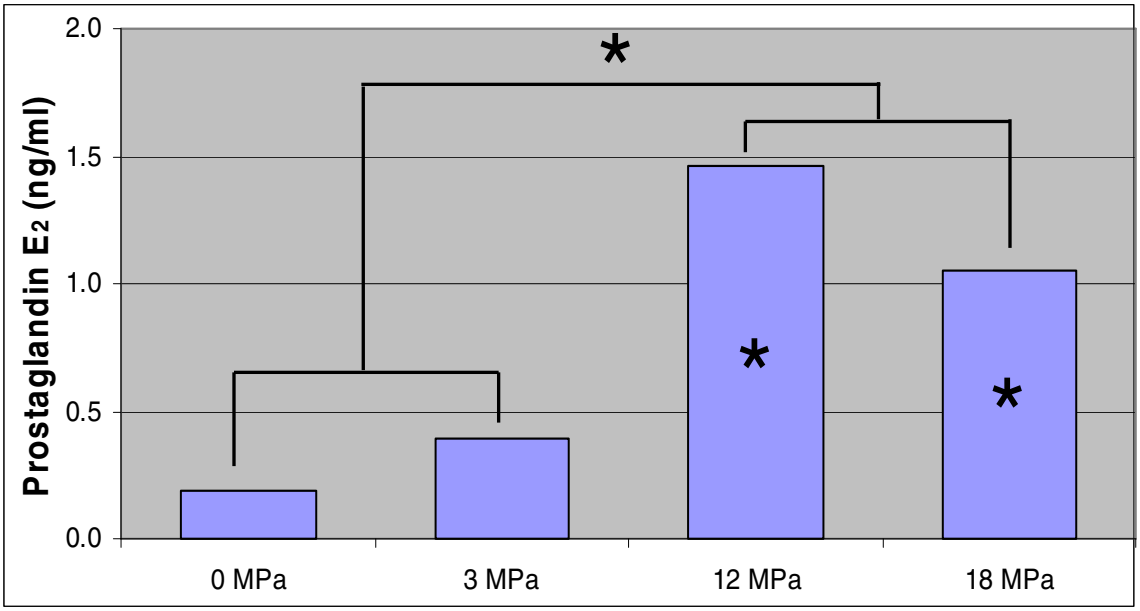

Figure 8: Raw levels of prostaglandin E2 in the media of tendons cyclically loaded for three days were load inducible and load-magnitude dependant. Asterisks on bars indicate a significant difference from the pre-loading level, while asterisks above bars represent significant differences between groups ($P < 0.05$) [30, 91].

The continuing question of PGE2's role in tendinopathy has led to several injection studies investigating the ability of PGE2 or PGE_1 to initiate degenerative changes similar to overuse tendinopathy. While histological studies suggested these injections could initiate degenerative changes [31], a recent study evaluating mechanical

properties found structural strength and stiffness to increase at 8 weeks after 4 weeks of injection [92]. Though this seems contradictory, perhaps PGE2 is activated for tissue remodeling which may be observed as degenerative in the short-term, yet improves material properties over time. The concept of PGE2 having a role in activating tissue remodeling has precedence in studies of bone, where increased bone mass has been observed following prostaglandin injections [93]. It has been suggested that tendon may experience a transient period of weakness as the tendon remodels, so excessive activation of remodeling by PGE2 may actually make the tissue more susceptible to further injury when it is being repetitively loaded [90]. Further investigation into the true effects of PGE2 in the short and long-term is needed.

2.5.2 PGE2 effects in cervical tissue

In the cervix, extensive remodeling occurs before the onset of parturition referred to as "ripening". This process allows gradual structural modification that will enable the cervix to stretch without breaking during labor. Similar to tendinopathy, the mechanisms involved in this process are not well understood. In a clinical setting, PGE2 and its analogs are used to induce the cervical ripening process and initiate labor because PGE2 production is increased in normal parturition [94-96].

Studies of the use of PGE2 gels to promote cervical ripening have suggested two main effects by which PGE2 may be acting to allow distension of the cervix: increased MMP activity and an increase in proteoglycan concentrations [75]. However, in a study of human cervical tissue, PGE2 treatment did not increase collagenase nor were typical collagen cleavage products indicative of enzymatic activity found in the samples [75].

Furthermore, biomechanical studies of PGE2 cervical ripening in the rat have suggested that changes associated with PGE2 treatment are not consistent with findings observed during exposure to collagenase. Buhimischi *et al.* compared physical and biomechanical characteristics of rat cervices exposed to PGE2 via gel *in vivo* and those cultured with collagenase *in vitro*, and found that the PGE2 exposure did not result in similar mechanical properties to those cervices cultured with collagenase. They found PGE2 increased plasticity, compliance and strength of the rat cervix, while collagenase had the opposite effect [74]. While some studies have found collagenase activity to be increased after PGE2 application to promote cervical ripening, the time period over which this activity is increased is relatively brief suggesting other mechanisms of lengthening may be occurring [94].

In vivo application of PGE2 to the cervix stimulates increases in proteoglycan concentration [97]. Due to the insufficient evidence that collagenase up-regulation is a main component of cervical ripening, it is possible that the increase in proteoglycan synthesis is a key factor in altering cervix mechanical properties. As in tendon, decorin binds to the collagen fibrils of the cervix, especially during the ripening process. It has been shown that the more decorin found within the extracellular matrix, the greater the rate of creep of the tissue [98].

A possible role of stromelysins, specifically MMP-3, in PGE2-induced cervical ripening has recently been noted. Increased collagenase mRNA levels seen at the onset of active labor fails to explain the progressive decrease in cervical tensile strength during the second half of gestation. These changes may be due to tissue remodeling that is mediated by MMP-3 [77]. PGE2 application in the cervix has not been shown to increase

MMP-3 expression or synthesis, but it has been shown to significantly increase the amount of active enzyme in the tissue [77].

PGE2, however, will not induce cervical ripening when applied mid-gestation in a rat model [77]. There is an increase in expression of the EP4 type of the four PGE2 receptors near term, and it is thought that ripening is mediated through activation of this particular receptor [99, 100]. Using specific agonists for EP1-4, it has been confirmed that the effects seen by PGE2 application in the cervix are only mimicked by activation of the EP4 receptor [82].

2.5 Clinical Treatment of Tendinopathy

Because of the relative mystery behind tendinopathy pathogenesis, there is not a definitive treatment. However, the first approach is generally to use nonoperative, conservative means to alleviate symptoms [27]. Focusing treatment to correct causative factors would be ideal.

2.5.1 Rest

With the structural damage incurred in tendinopathy, rest may allow time for the tendon to repair partial tears. However, tendon disuse does have negative consequences, including lowered metabolic activity, increased collagen turnover, reduced cross-linking, collagen disorientation, and decreased GAG content [1, 16, 101]. This can result in mechanical property changes, i.e. decreased tensile strength, elastic stiffness, total weight and energy to failure [1, 13-16].

2.5.2 Exercise

If the pain associated with a patient's tendon disorder is not too severe, a simple decrease in frequency, intensity and duration of the aggravating activity may suffice [27]. Controlled mobilization has been shown to enhance tendon strength properties, so loading below damage levels can be beneficial during healing [15, 102].

2.5.3 Pharmaceutical Treatment

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most frequently used pharmacologic substance in treating tendinopathy [27]. NSAIDs are known to reduce pain associated with tendon disorders [103]. There is much contradictory evidence about the efficacy of NSAID use, as well as considerable data outlining the potential harms of these drugs.

NSAIDs inhibit the enzyme COX, and thus inhibit prostaglandin synthesis, which results in analgesic and anti-inflammatory effects. COX-1 is constitutively expressed in many tissue types, and has a variety of physiologic roles including maintaining gastric mucosa, regulating renal blood flow, and influencing platelet aggregation. COX-1 inhibitors have been implicated in gastrointestinal bleeding and ulcering. No benefits of COX-1 inhibition from NSAIDs have been seen [104]. The pathway on which NSAIDs act is shown in Figure 9.

Meanwhile, COX-2 is mainly an inducible enzyme present exclusively during inflammation. Discovery of this isoform led to development of COX-2 specific inhibitors to effectively block inflammation and pain without disturbing normal tissue functions. Initially, these drugs appeared to function with significantly fewer serious gastrointestinal adverse effects than non-selective NSAIDs [54]. However, over time coxibs have been associated with changes in blood pressure that lead to increased risk of

heart attack, thrombosis and stroke. For this reason, certain coxibs have been banned or permanently withdrawn from the market.

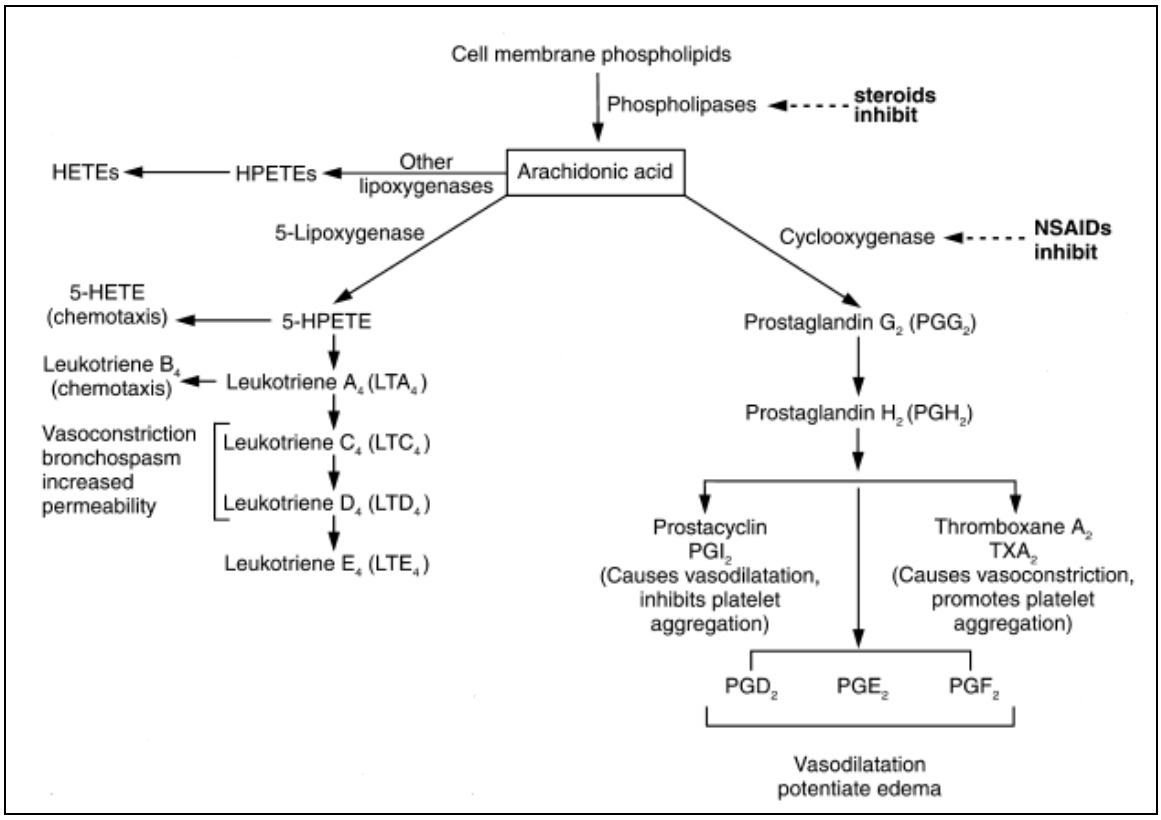

Figure 9: The points of action of pharmaceutical tendinopathy treatments in the arachidonic acid cascade [4]

Corticosteroids are another commonly used treatment in musculoskeletal conditions. They can be injected directly in the tendon sheath to alleviate inflammation by blocking both COX and lipoxygenase pathways of arachidonic acid metabolism.

2.6 Tendinopathy Models

2.6.1 *In vivo* animal and human studies

Animal models of tendinopathy can either attempt to induce degenerative changes through physical or chemical means. Physical attempts to model tendinopathy require exercising an animal with a high enough frequency over a long enough period of time to induce overuse changes in the tendon. For example, rabbit hind paws were passively exercised for 2 hours, 3 times a week, for up to 6 weeks. This caused the Achilles tendon to show increased numbers of capillaries, increased inflammatory cell infiltration, edema, and fibrosis [105]. Overuse has also been modeled in rats, by engaging the animals in a reaching task 2 hours a day, 3 times a week, for up to 8 weeks. This model showed an increase in macrophage infiltration of the upper-extremity tendons, and the collagen fibrils becoming frayed [106]. Finally, treadmills have also been used to exercise rats. This study showed a decrease in supraspinatus tendon cell number, as well as disorganization and damage of collagen fibers at 8 weeks and a reduction in stress at failure [22].

These animal models of tendinopathy have shown that repetitive mechanical loading causes inflammation in the tendon. The investigators conclude that destruction of the tendon is due either to mechanical damage, biochemical mediators, or both. However, because exercise animal models for tendinopathy can have a high cost and show inconsistent results due to the inability to precisely control and normalize the loads seen in the tissues, the use of injection animal models of tendinopathy has been investigated.

In one animal model, PGE_1 was injected into the peritendinous space of the rat patellar tendon, and was shown to induce inflammation and degeneration in and around the tendon [91]. Likewise, a study that injected PGE2 into the midsubstance of rabbit patellar tendons induced profound changes in the tendon extracellular matrix [30]. However, previous work in our laboratory showed varying results. Rat patellar tendons were injected each week, for 4 weeks, with PGE2. The tendons showed improved biomechanical strength and stiffness at 8 weeks [31]. These results show that more investigation into an appropriate injection model protocol is needed to ensure a more accurate *in vivo* simulation of tendinopathy.

2.6.2 *In vitro* culture studies

Due to the difficulty controlling tendon loading *in vivo*, several *in vitro* models have been developed to quantify tissue changes during stress deprivation, cyclic loading, and overuse. The changes seen both biochemically and mechanically in tendon with and without loading can be assessed in cell or tissue culture.

A basic model of the cellular mechanisms that may be implicated in tendinopathy can be achieved by cyclically loaded cells in culture. Fibroblasts cultured on a flexible substrate that can be subjected to stretching have shown changes in protein expression and synthesis, as well as changes in release of various cytokines and inflammatory molecules. Thus, these factors might be considered to have a role in the development and modulation of injury [107-112]. However, without the extracellular matrix, tissue properties cannot be assessed.

Tissue explant models can examine the cellular responses of tendon fibroblasts to mechanical loads, as well as determine the effect of those responses on the extracellular

matrix. By utilizing a tissue loading device, tendons can be exposed to stress deprivation conditions, static loads or cyclic loads and changes in the biochemical and mechanical properties can be measured [90, 113-116]. The main advantage of these types of systems is that loading can be controlled, which allows for more precise determination of the effects of loading on tendon.

Chapter 3

RESEARCH DESIGN AND METHODS

3.1 Research Design Specific Aims

AIM 1: Design and fabricate a device to allow static loading and creep measurement of viable rat tail tendon fascicles submerged in media and housed in an incubator.

- **Hypothesis 1: System will allow for short term creep studies by being able to statically load rat tail tendon and monitor changes via strain analysis during loadings**.

AIM 2: To evaluate the mechanical response (strain accumulation and mechanical properties) of rat tail tendon fascicles to PGE2 exposure while under 0.3 MPa static load and unloaded.

- **Hypothesis 2.1: Tendons will require at least 24 hours of PGE2 exposure in culture in order to display effects.**
- **Hypothesis 2.2: PGE2 will increase creep in culture while maintaining failure stress levels as compared to untreated tendons.**

AIM 3: Determine if MMPs have a role in the mechanical response of tendon to PGE_2 exposure.

- **Hypothesis 3.1: Effects of PGE_2 will be reversed by addition of a broad-spectrum MMP inhibitor to the system.**

- **Hypothesis 3.2: Effects of PGE_2 will be reversed by addition of an MMP-3 inhibitor.**

3.1.1 Design and Fabrication of the Tendon Loading Apparatus

In order to statically load rat tail tendon fascicles, there were considerable design constraints. The tendon ends must ideally be unsubmerged for gripping, while the midsubstance is submerged in treated media. With this constraint we determined that the tendons should be cultured in a glass Pasteur pipette, as described by Wood, *et al.* containing the experimental media. However, we improved upon this method by threading through a rubber stopper from a 7.0 ml Vacutainer tube order to have a substantial base for static loading in culture. This stopper housed a tube prepared from a Pasteur pipette, which was then able to store media during culture.

The end of the tendon fascicle was allowed to dry for 15 minutes and the dried end was threaded through the eye of a straight needle. The needle with fascicle was then inserted longitudinally through the center of the stopper. The stopper was then placed on the glass tube and the tube filled with media. This setup ensured the midsection was exposed to experimental media while the section for clamping was dry and isolated from the media A drawing of the culture tube setup is illustrated in Figure 10.

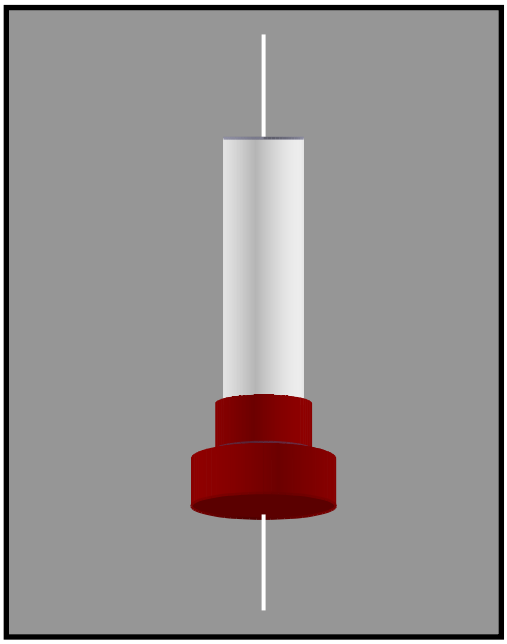

Figure 10: Schematic drawing of tube-stopper apparatus with tendon fascicle pulled through

Once the culture tubes were designed, the loading apparatus could be developed. The design criteria identified were: easy to assemble, disassemble, sterilize, use within a biological incubator, and house our laboratory's preexisting calibration rods for use with our visual strain analysis system to measure tendon lengthening in culture. The device was machined by the UNC Physics Instrument Shop out of aluminum based on our design (Appendix A).

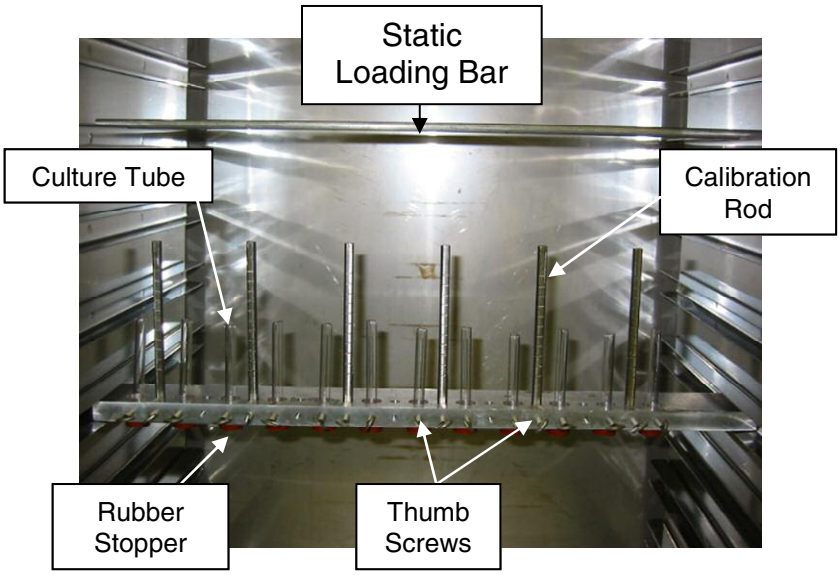

Figure 11: Incubator setup with static tissue loading device

Figure 11 shows the static loading device. It has the capability to house 12 individual tubes with stoppers as well as one calibration rod per sample. The loading bar is placed directly above the tubes, and fish weights are draped over the rod with fishing line until the desired stress level is achieved in the tissue.

Tendon fascicles were loaded in this apparatus by thumb screws holding the Vacutainer tube stoppers in place with the media-containing culture tubes upright. The end of the fascicle above the culture media was dried out as previously mentioned, and was then affixed with Loctite super glue between two 1 cm^2 silicone rubber pads. Also affixed between these pads was the end of a fishing line that attached on the opposite end to a small weight of approximately 14 g which was then draped over the static loading bar to apply an approximate 0.3 MPa load to the fascicle. This load level was determined in pilot studies that showed control tendon fascicles under 0.3 MPa load for 16 hours acquired about a 5% strain.

3.1.2 Tissue Culture Methods

Using the static loading device, experiments to measure accumulated strain were carried out. Tissue was obtained from untreated Sprague Dawley rats sacrificed for other uses. In order to control the variability between rat sources, only female Sprague Dawley retired breeder rats within the weight range of 300-400 g were used in these experiments.

Tendon fascicles were isolated by removing the tail from the animal via an incision between the two most proximal vertebrae. The opposite end of the tail was cut between two more distal vertebrae, approximately 5 cm proximally from the distal tip. Individual fascicles were teased from the tendons out the distal end of the tail.

Isolated fascicles were hydrated in a sterile media bath. Fascicles of similar size were isolated and laid taut across a Petri dish. This resulted in both fascicle ends becoming dried out, while the fascicle midsubstance remained submerged in sterile media. Diameters were measured using an eye-piece micrometer on an Olympus Tokyo CK inverted microscope; only tendons with cross-sectional areas of 370 ± 25 µm were used in the study. Once fascicles were isolated and measured, they were threaded through a glass tube and rubber stopper using a sterile straight sewing needle as described in section 3.1.1. The tube was then filled with 1.5 ml sterile media without any experimental additives. The tissue was allowed to equilibrate for two hours in an incubator before this media was aspirated and replaced with experimental media.

Tissue culture medium for all groups consisted of phenol-red free DMEM-H media (pH 7.2) supplemented with 20mM Hepes, 100IU/ml penicillin, 100µg/ml streptomycin, 2µg/ml amphoteracin B, 100µM ascorbate, 1mM sodium pyruvate, and

4mM L-glutamine. The final media additive was 2μM arachidonic acid, a supplement to insure adequate substrate for the production of prostaglandins. Additionally, experimental groups received either 1μg/ml prostaglandin E_2 (Cayman Chemicals) alone, or in combination with 5μM of a broad range MMP inhibitor (Chemicon) or 50μM specific MMP-3 inhibitor (EMD Biosciences).

For Aim 2 experiments, the rat tail tendon fascicles were randomly divided into two loading regimen groups: 1) loaded and 2) unloaded, and two treatment groups: 1) control and 2) PGE_2. The loaded groups underwent 24 hours of unloaded culture before the introduction of a 0.3 MPa static tensile load for 16 hours. Load was applied after 24 hours of culture by hanging a 14 gram fish weight over the tendon-hanging bar. The unloaded group remained unloaded for the entire 40 hour culture period and underwent material property testing at the following timepoints: 0 hour (immediately following harvest), 6 hour, 12 hour, 24 hour, and 40 hour. Material property testing methods will be subsequently described. Sample sizes were as follows: Loaded, control group contained 10 fascicles; loaded, PGE_2-treated fascicles contained 8 fascicles; unloaded, control fascicles contained 10 fascicles; and unloaded, PGE_2-treated fascicles contained 9 fascicles.

Two blue #6-0 sutures were used as strain markers affixed via Loctite gel super glue at the top of the tube and on the dried tissue above solution. Strain was measured either using a visual strain analysis system (VSA) [117] or digital microcalipers initially after the preculture media was added, then immediately after the experimental media was introduced, after 24 hours of unloaded culture (beginning of static load), and after 16 hours of static load. Figure 12 shows a close-up image of the tendon fascicles in the

static loading device during culture. The initial gage length of the entire tissue was measured, and then deformation was measured subsequent to this with a reduced field of view containing the strain markers and calibration rods in order to increase the resolution of the VSA system.

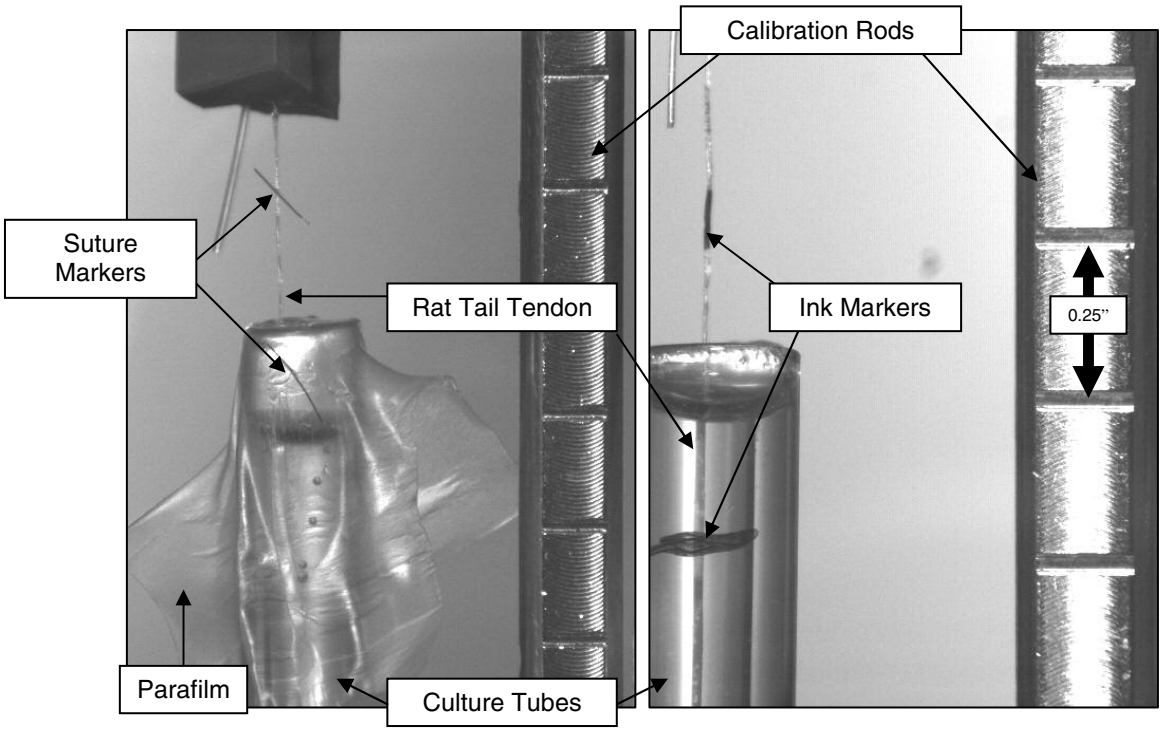

Figure 12: Rat tail tendons during statically loaded culture with either suture markers or ink markers and calibration rods in order to measure strain using a camera and visual strain analysis program

The experiments of Aim3 investigated the role of MMP activity in the tissue effects induced by PGE2 exposure. For this Aim, tendon fascicles in all groups were cultured unloaded for 40 hours. Experiments consisted of 4 treatment groups: 1) Control (n=15), 2) PGE2 (1µg/ml) (n=10), 3) PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM, n=12), and 4) PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM, n=12). After 40 hour in unloaded culture, the tendon fascicles underwent material property testing.

Immediately following culture, fascicles in tubes were placed in a 4ºC refrigerator until mechanically loaded by a servo-hydraulic materials testing system (8500 Plus, Instron Corporation, Canton, MA, USA). Before testing, the tendon was cut from the stopper and affixed with Loctite gel super glue between two square, silicone rubber grips. The tissue was wrapped in saline-soaked gauze until loaded into the grips of the tensile tester. Figure 13 illustrates the failure test setup, which was conducted completely submerged in a PBS bath at room temperature. The failure test regimen began with pre-conditioning ten cycles of a 1% strain, 0.5 Hz haver-sine waveform prior to failure testing with a 1% strain/sec linear ramp. Load cell (Model 31, Sensotec, Columbus, OH) and grip strain data (maximum nonlinearity error of 0.2% of full scale +/- 75mm LVDT) were gathered during the failure loading for structural and material property analysis.

Instron Series IX software was utilized to calculate maximum load, maximum stress, strain at maximum load, energy at maximum load and Young's modulus during the failure test was calculated. The Young's modulus calculation was made via a linear regression between the limits of 25% and 75% of the maximum load.

Figure 13: Instron failure testing setup

3.1.3 Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's mean comparison testing was utilized to evaluate differences in mechanical properties between treatment groups. Two-way ANOVA was utilized to evaluate the mechanical property changes

caused by PGE2 treatment over time. A significance level of 0.05 was used for these tests.

Chapter 4

RESULTS

4.1 PGE2 Timecourse

Rat tail tendon fascicles were cultured, unloaded, with or without 1 μg/ml PGE2 for 40 hours. The tissue material properties were measured in a tensile failure test immediately (time 0), and then at 6 hours, 12 hours, 24 hours and 40 hours.

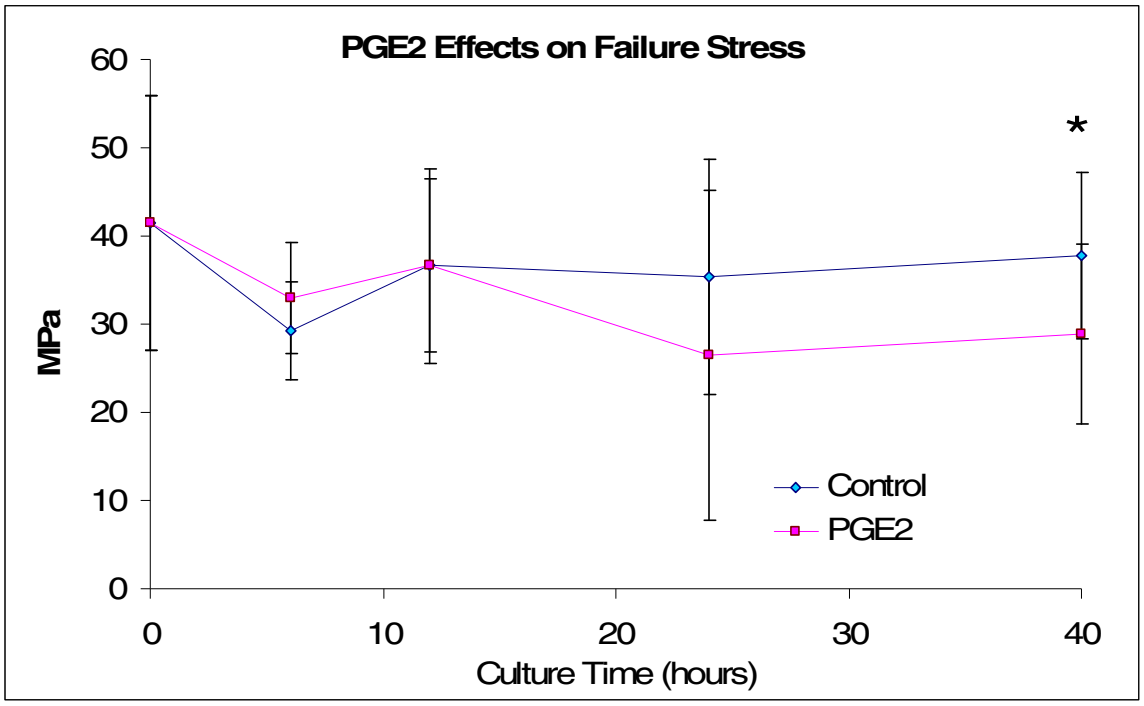

Figure 14: Maximum stress value reached during tensile failure test of tendon fascicles cultured with and without PGE2 unloaded for 40 hours. Error bars indicate standard deviations. Asterisks represent significant differences between groups (2-way ANOVA, Tukey's test for multiple comparisons, $P < 0.05$). Sample sizes were as follows: Loaded, control group contained 10 fascicles; loaded, PGE_2-treated fascicles contained 8 fascicles; unloaded, control fascicles contained 10 fascicles; and unloaded, PGE_2-treated fascicles contained 9 fascicles.

The PGE2 and control groups show no significant differences in stress at failure before the 40 hour time point. At 40 hours, the PGE2-treated tissue displayed a 23.5% decrease in average stress level at failure as compared to control (Figure 14).

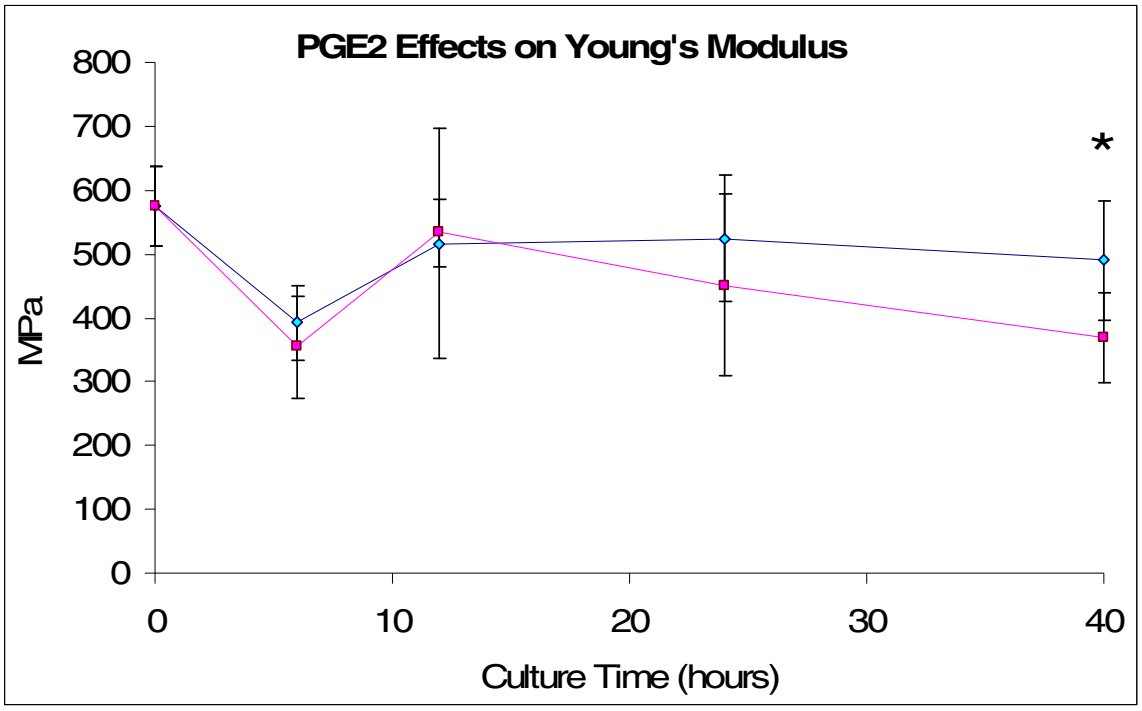

Figure 15: Young's modulus measured during tensile failure test of rat tail tendon fascicles cultured with and without PGE2 unloaded for 40 hours. Error bars indicate standard deviations. Asterisks represent significant differences between groups (2-way ANOVA, Tukey's test for multiple comparisons, $P < 0.05$). Sample sizes were as follows: Loaded, control group contained 10 fascicles; loaded, PGE_2-treated fascicles contained 8 fascicles; unloaded, control fascicles contained 10 fascicles; and unloaded, PGE_2-treated fascicles contained 9 fascicles.

PGE2's effects on tendon fascicle stiffness were measured via calculation of Young's modulus. As with stress at failure, stiffness levels of PGE2-treated tissue did not show a significant decrease before the 40 hour time point. At the 40 hour time point, the average modulus of the PGE2 group was 24.5% lower than that of controls (Figure 15).

The strain to failure for both PGE2-treated and control tendon fascicles was unchanged throughout the 40 hour culture (Figure 16). Likewise, energy measurements

show no significant differences between PGE2 and control groups at any point during the time course study (Figure 17).

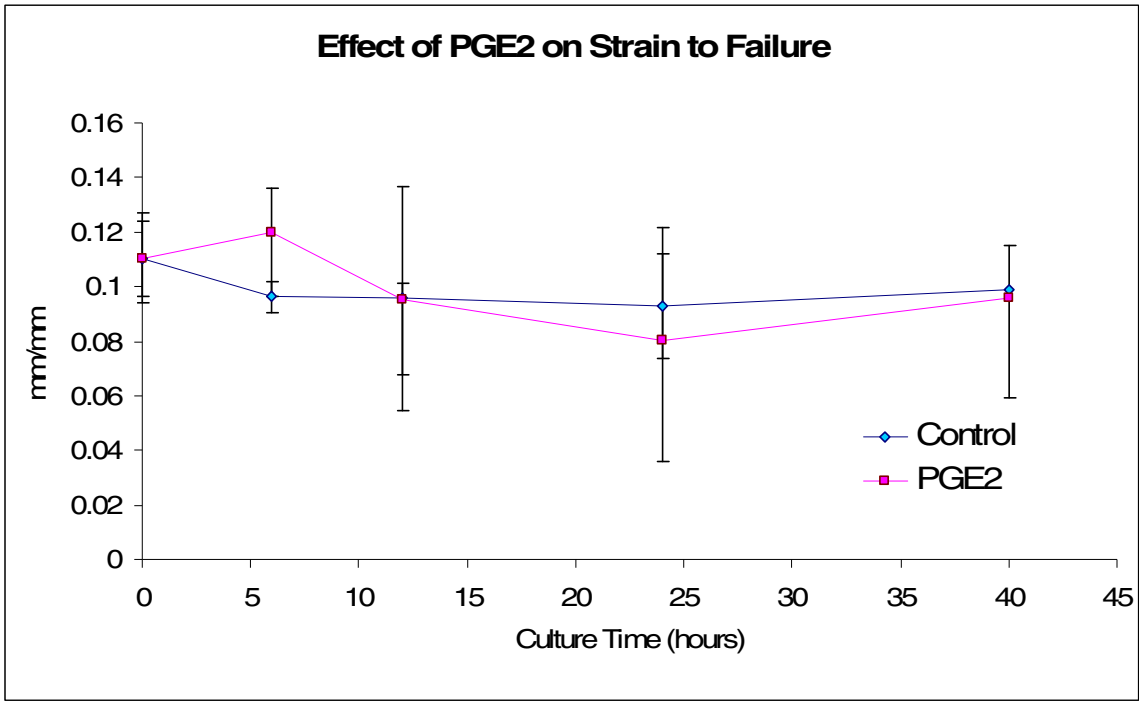

Figure 16: Strain measured during tensile failure tests of rat tail tendon fascicles cultured with and without PGE2 unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Loaded, control group contained 10 fascicles; loaded, PGE_2-treated fascicles contained 8 fascicles; unloaded, control fascicles contained 10 fascicles; and unloaded, PGE_2-treated fascicles contained 9 fascicles.

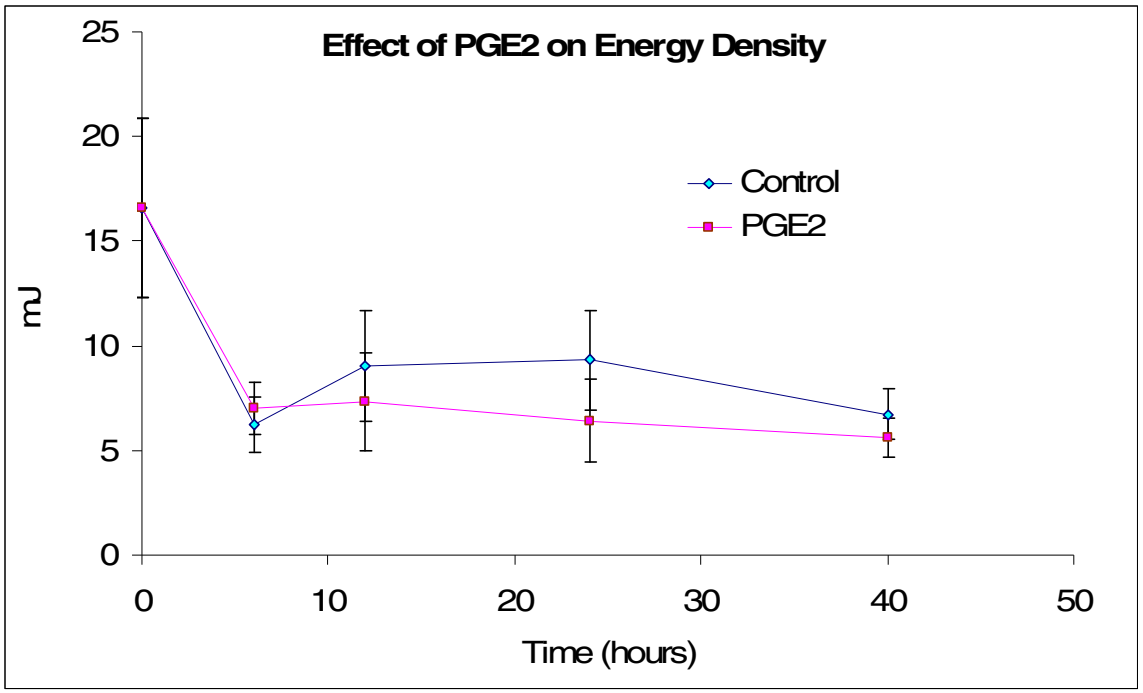

Figure 17: Energy measured during tensile failure tests of rat tail tendon fascicles cultured with and without PGE2 unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Loaded, control group contained 10 fascicles; loaded, PGE_2-treated fascicles contained 8 fascicles; unloaded, control fascicles contained 10 fascicles; and unloaded, PGE_2-treated fascicles contained 9 fascicles.

4.2 Statically Loaded Tendon

After characterization of PGE2's effects in rat tail tendon fascicles over the culture time period, the tendons were then exposed to static load in culture. All fascicles were cultured, unloaded, with or without 1μg/ml PGE2 for 24 hours, and then were loaded with an approximate 0.3 MPa load for 16 hours. Tissue lengths were measured for strain calculation at four time points: 1) initially after harvest, 2) after experimental media addition, 3) after 24 hours at the beginning of static load application, and 4) after 16 hour static load.

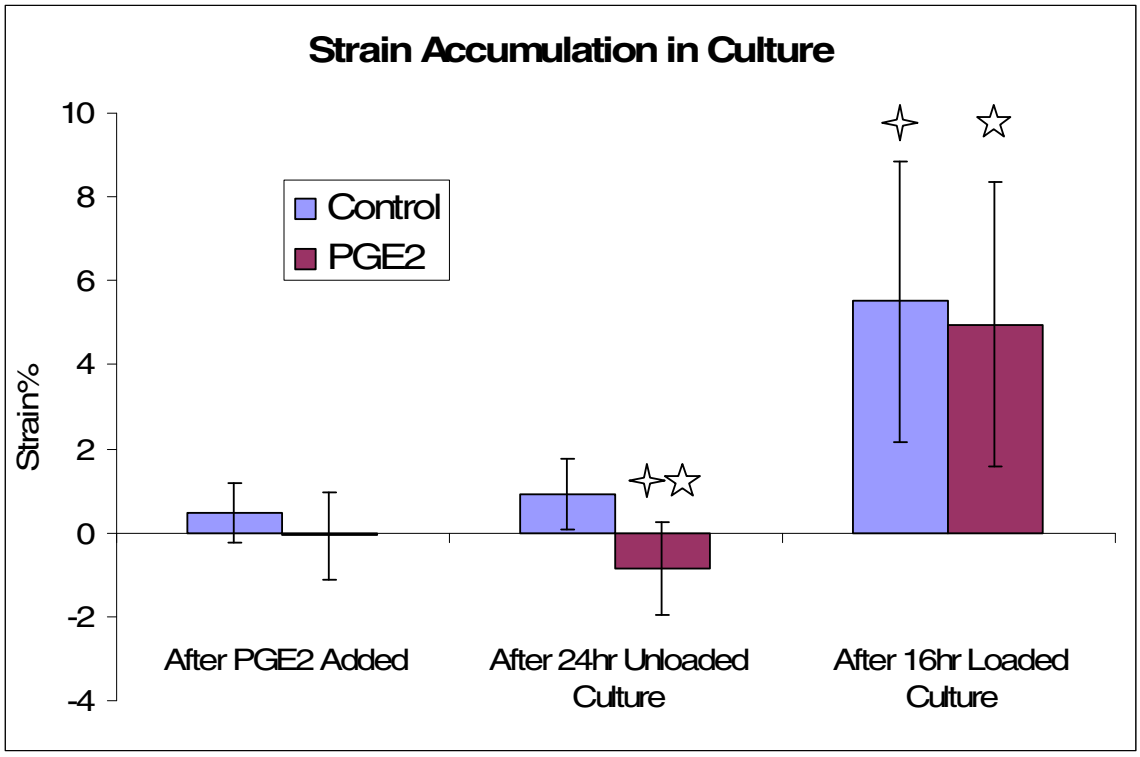

Figure 18: Strain measured before and after 0.3 MPa static load applied in culture to rat tail tendon fascicles treated with or without PGE2 and unloaded for 24 hours followed by 16 hour static load. Error bars indicate standard deviation. 4-point stars represent significant difference from own first strain measurement, and 5-point stars represent significant differences from control at that same time point (2-way ANOVA, Tukey's test for multiple comparisons, $P < 0.05$). Sample sizes were as follows: Loaded, control group contained 10 fascicles; loaded, PGE_2-treated fascicles contained 8 fascicles; unloaded, control fascicles contained 10 fascicles; and unloaded, PGE_2-treated fascicles contained 9 fascicles.

No strain accumulation differences were observed between control and PGE2 groups during initial preculture before experimental media was introduced. During the 24 hour unloaded culture, the PGE2-treated fascicles accumulated a negative strain which is significantly different from the positive strain accumulation seen in the control group at the same time point (Figure 18). After the 16 hour static load, PGE2-treated and control tendons show no difference in the amount of strain accumulated (Figure 18). The significant differences seen in mechanical properties of failure stress and Young's Modulus between the PGE2 and control tendons after 40 hours in unloaded culture were eliminated with the introduction of a 16 hour static load (Figure 19 and 20). Failure strain and energy to failure were not significantly effected by either treatment or loading regimen (Figures 21 and 22).

Figure 19: Maximum stress value reached during tensile failure test of rat tail tendon fascicles cultured with and without PGE2 (1µg/ml) and either unloaded for 40 hour culture time, or unloaded for 24 hours followed by 16 hour 0.3 MPa static load in culture. Error bars indicate standard deviation. Asterisks represent significant differences between groups (1-way ANOVA, Tukey's test for multiple comparisons, $P < 0.05$). Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

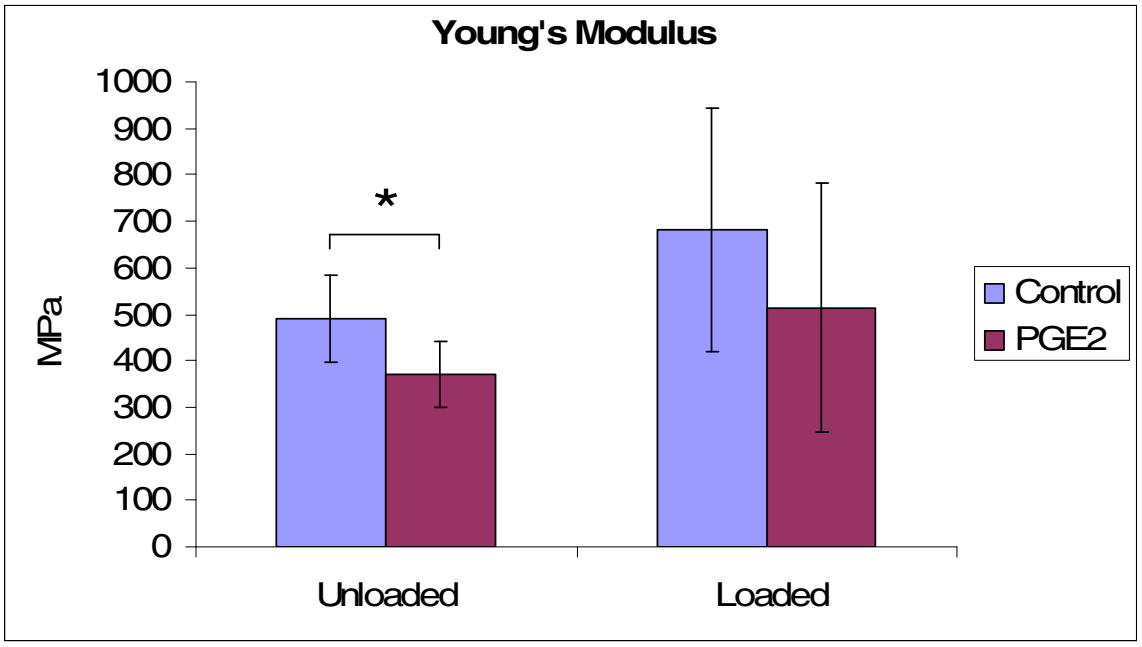

Figure 20: Young's Modulus measured during tensile failure test of rat tail tendon fascicles cultured with and without PGE2 (1µg/ml) and either unloaded for 40 hour culture time, or unloaded for 24 hours followed by 16 hour 0.3 MPa static load in culture. Error bars indicate standard deviation. Asterisks represent significant differences between groups (1-way ANOVA, Tukey's test for multiple comparisons, P < 0.05). Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12).

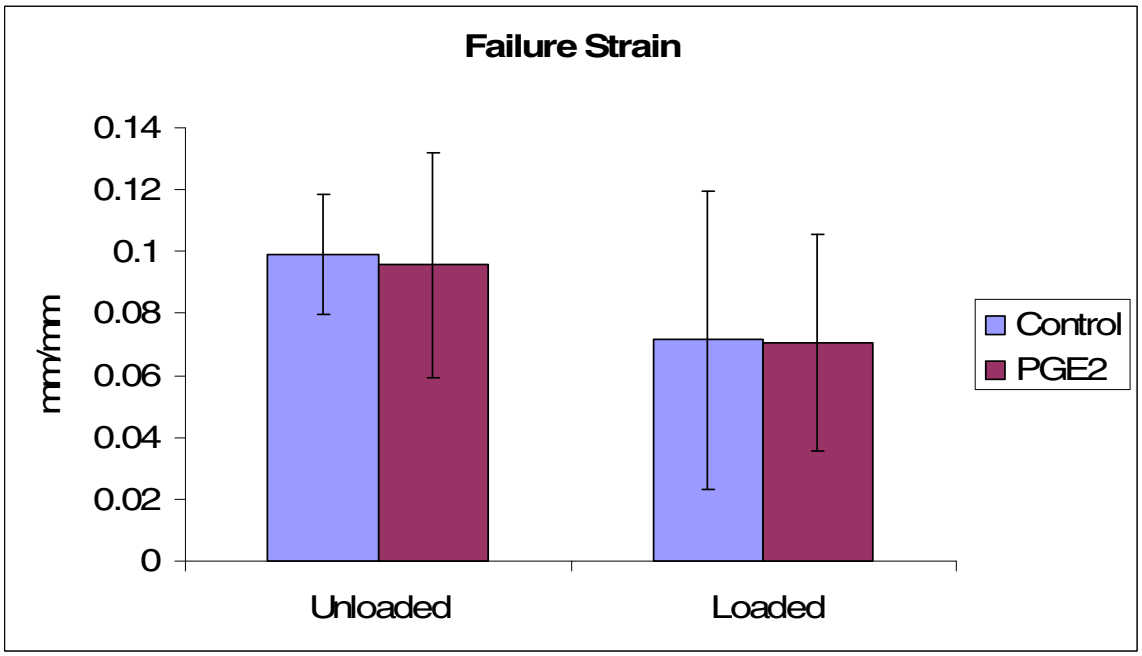

Figure 21: Strain measured during tensile failure test of rat tail tendon fascicles cultured with and without PGE2 (1µg/ml) and either unloaded for 40 hour culture time, or unloaded for 24 hours followed by 16 hour 0.3 MPa static load in culture. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12).

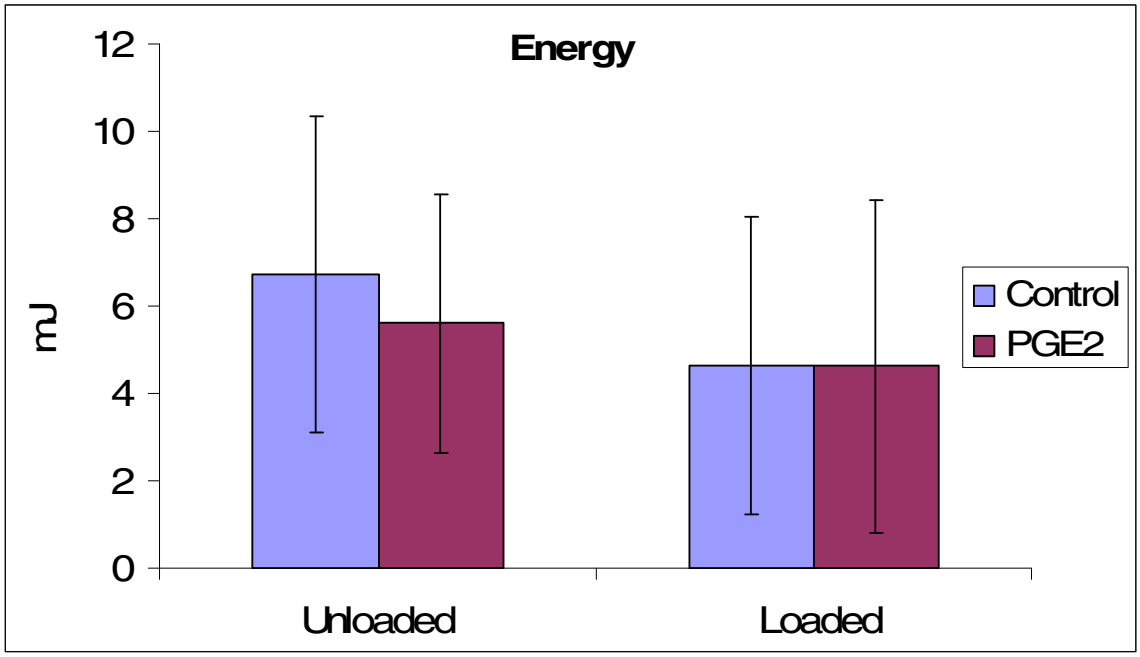

Figure 22: Energy measured during tensile failure test of rat tail tendon fascicles cultured with and without PGE2 (1µg/ml) and either unloaded for 40 hour culture time, or unloaded for 24 hours followed by 16 hour 0.3 MPa static load in culture. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12).

4.3 Nonspecific MMP inhibition

A broad-spectrum MMP inhibitor was introduced to the unloaded culture system in order to determine MMPs role in PGE2-induced changes in mechanical properties. When PGE2-treated fascicles were also cultured with the MMP inhibitor, their failure stress levels were no different from controls after 40 hours. The tendons treated with PGE2 alone displayed a significant decrease in failure stress levels when compared to both control groups and the group treated with PGE2 plus MMP inhibitor (Figure 23).

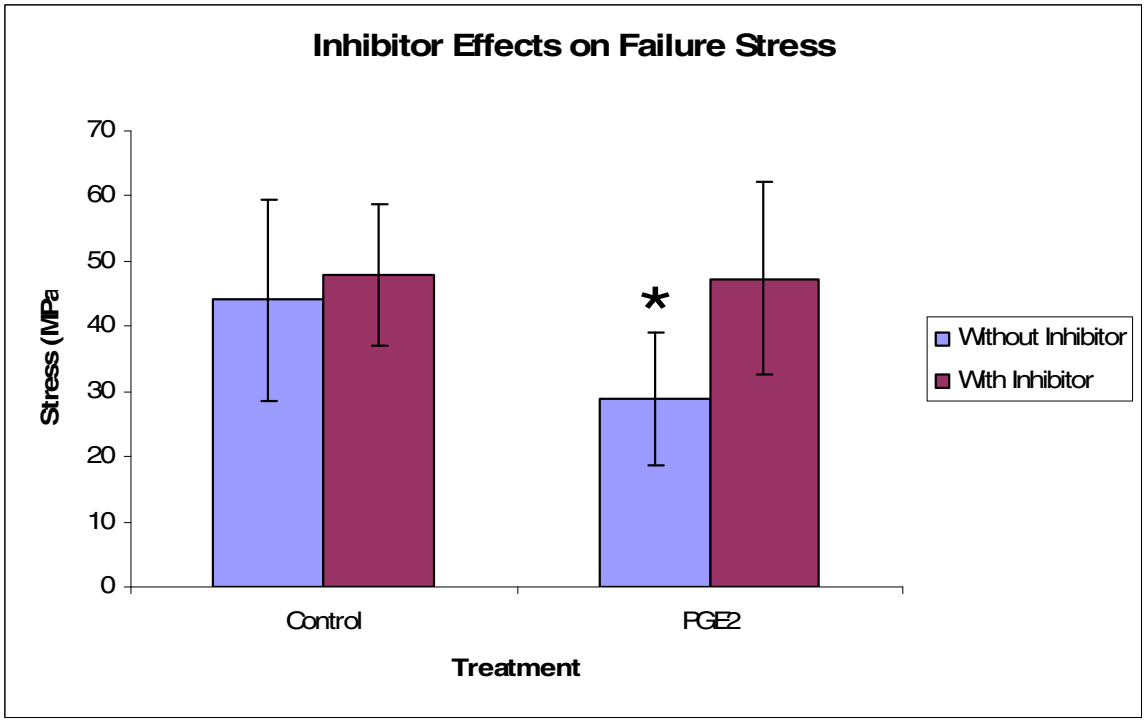

Figure 23: Maximum stress value reached during tensile failure test of rat tail tendon fascicles cultured with and without PGE2 (1µg/ml) and with or without 5 µM of a broad-spectrum MMP inhibitor unloaded for 40 hours. Error bars indicate standard deviation. Asterisks represent significant differences between groups (1-way ANOVA, Tukey's test for multiple comparisons, $P < 0.05$). Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

The MMP-inhibitors effects on tissue stiffness after a 40 hour unloaded culture were similar to the effects on failure stress. Tendon tissue treated with PGE2 alone showed a significant decrease in stiffness as compared with the control group or the PGE2 plus inhibitor group (Figure 24). The MMP inhibitor caused the tissue stiffness in the PGE2-treated group to remain at the same level as controls.

The broad-spectrum MMP inhibitor did not have any significant effect on the strain to failure during the tensile failure test in any group (Figure 25). However, the inhibitor's effects on energy density was similar to its effects on failure stress and Young's modulus, in that the inhibitor maintained energy levels similar to controls, while PGE_2 caused a significant decrease in energy during the tensile failure test (Figure 26).

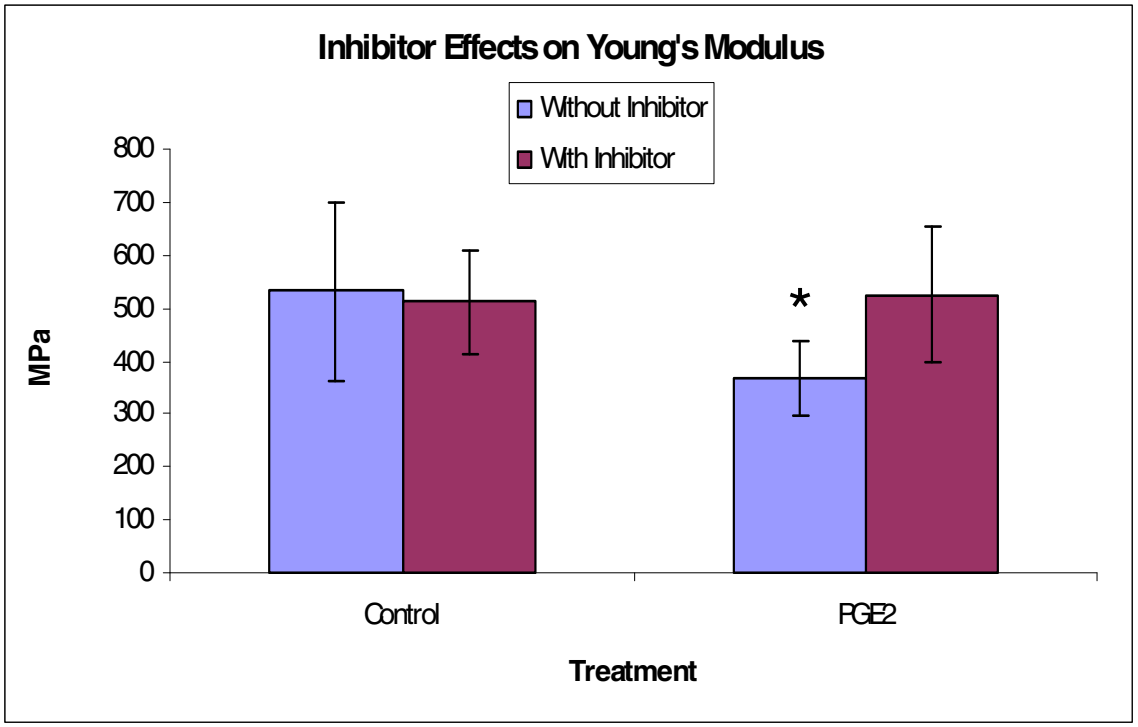

Figure 24: Young's modulus measured during tensile failure test of rat tail tendon fascicles cultured with or without PGE2 (1µg/ml) and with or without 5 µM of a broad-spectrum MMP inhibitor unloaded for 40 hours. Error bars indicate standard deviation. Asterisks represent significant differences between groups (1-way ANOVA, Tukey's test for multiple comparisons, $P < 0.05$). Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

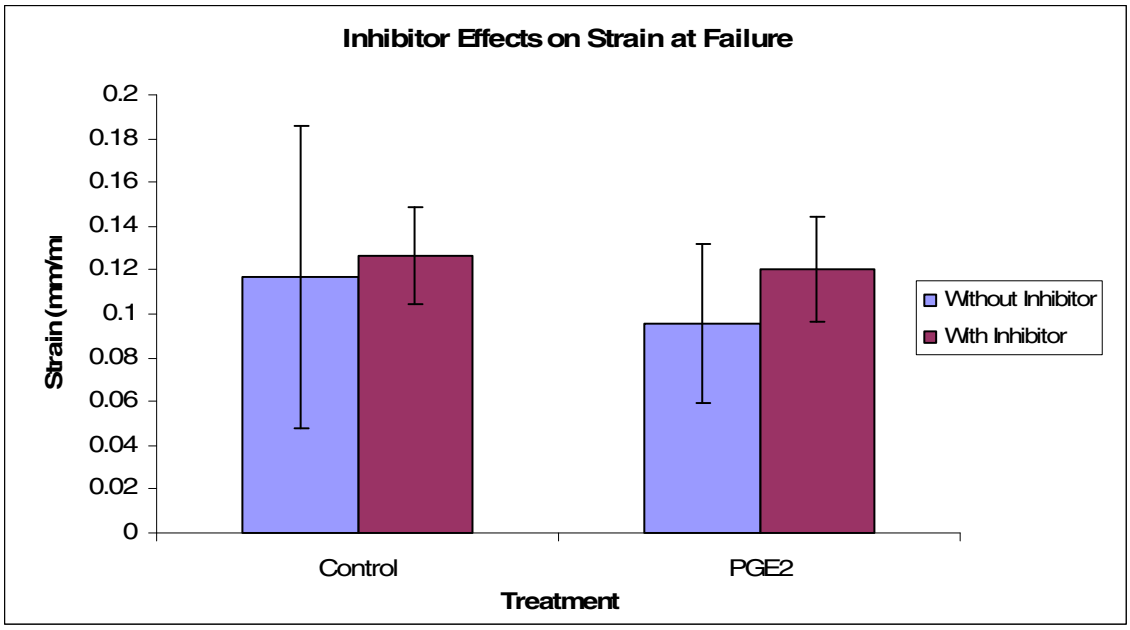

Figure 25: Strain measured during tensile failure test of rat tail tendon fascicles cultured with or without PGE2 and with or without a broad spectrum MMP inhibitor (5µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

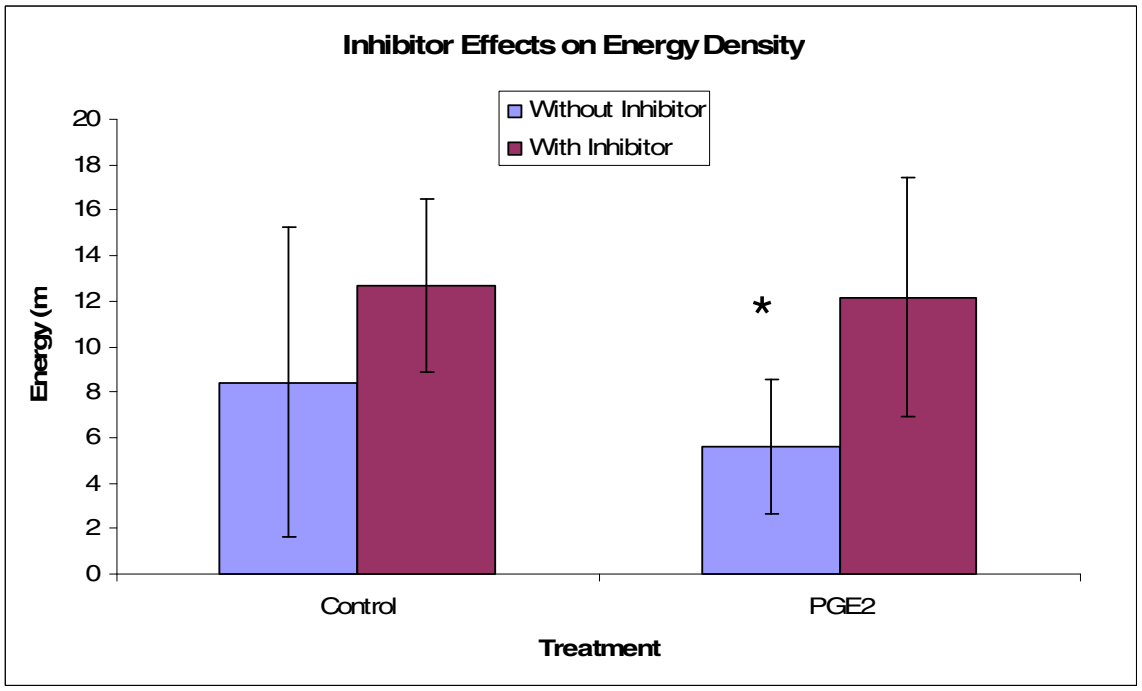

Figure 26: Energy measured during tensile failure test of rat tail tendon fascicles cultured with or without PGE2 (1µg/ml) and with or without 5 µM of a broad-spectrum MMP inhibitor unloaded for 40 hours. Error bars indicate standard deviation. Asterisks represent significant differences between groups (1-way ANOVA, Tukey's test for multiple comparisons, $P < 0.05$). Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

4.4 Specific MMP-3 Inhibition

Rat tail tendon fascicles were cultured with or without 1 µg/ml PGE2, and those cultured with PGE2 were also cultured with or without 50 µM of a specific MMP-3 inhibitor, for 40 hours in unloaded culture. Immediately following culture, the tissue was loaded to failure in a tensile failure test to obtain strength, strain, stiffness and energy measurements.

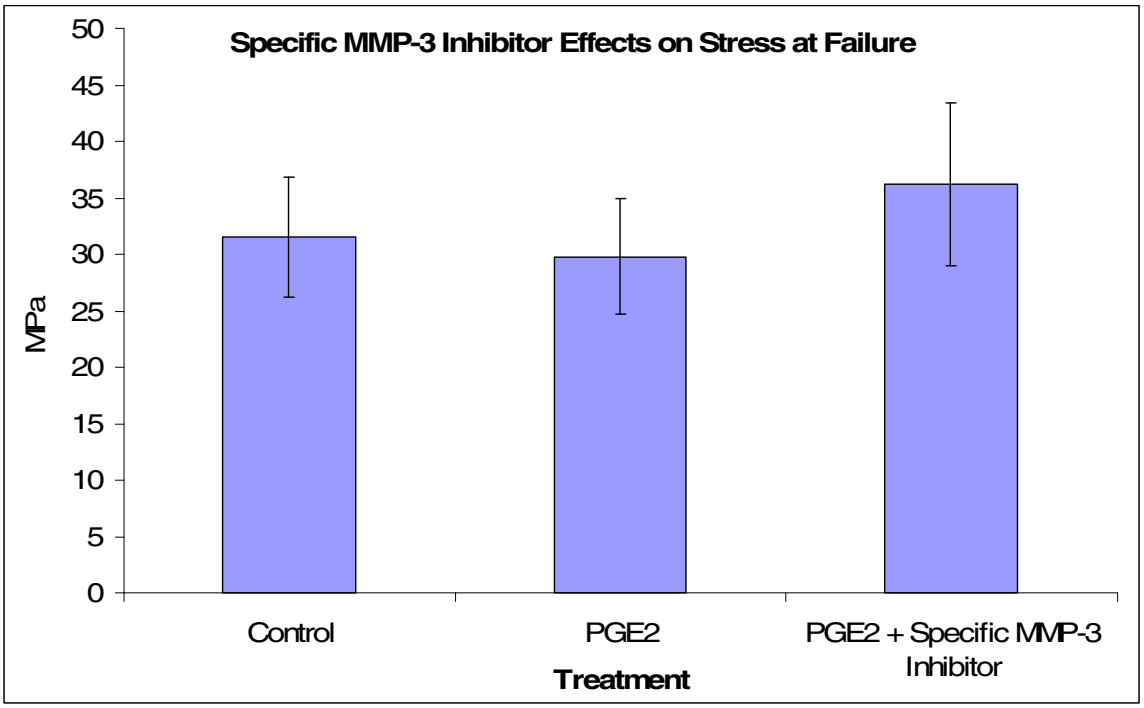

Figure 27: Maximum stress value reached during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor (Control), with PGE2 only, or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

No significant differences were found in the stress measured at failure between the groups (Figure 27). However, there was a trend for PGE2 to decrease the stress seen at failure and for the specific MMP-3 inhibitor to increase the stress in the tendon at failure. Tendon stiffness was unaffected by any of the treatment groups (Figure 28). The specific MMP-3 inhibitor tended to increase the strain to failure and energy over control

and PGE2-treated tendons, though these increases were not significantly different (Figures 29 and 30).

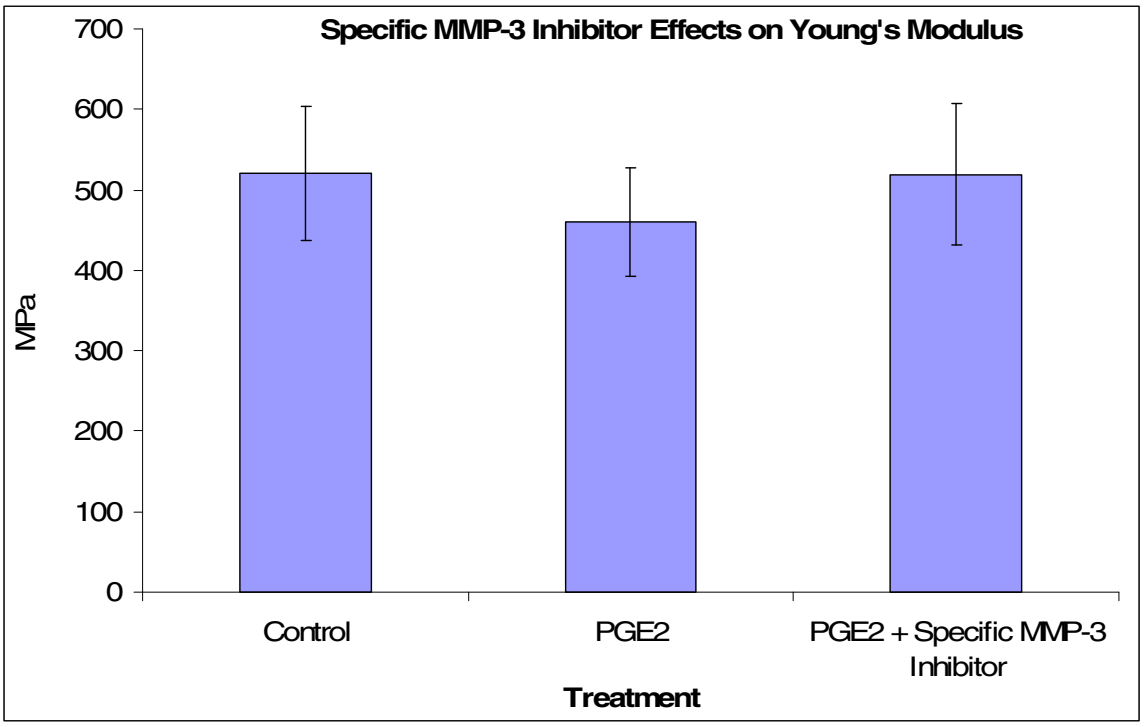

Figure 28: Young's modulus measured during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor (Control), with PGE2 only, or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

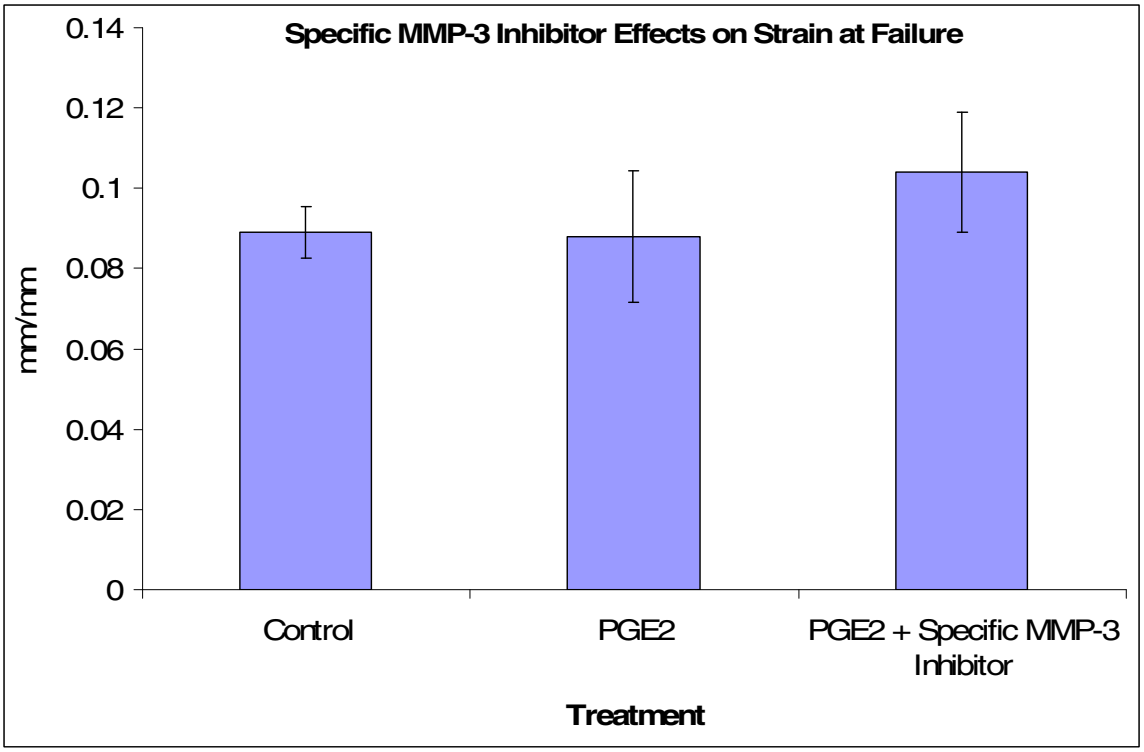

Figure 29: Maximum strain measured during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor (Control), with PGE2 only, or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

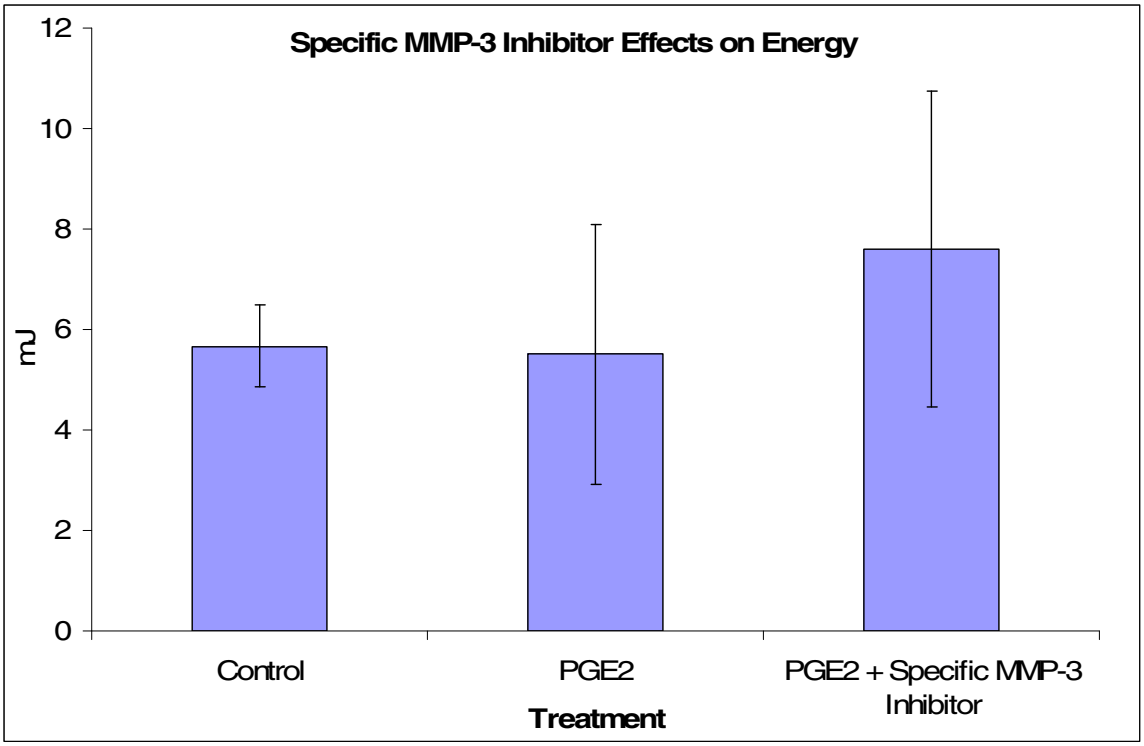

Figure 30: Energy measured during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor (Control), with PGE2 only, or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) (n=10), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

The effects of the broad-spectrum MMP inhibitor (Ilomastat) and the specific MMP-3 inhibitor were compared by culturing rat tail tendon fascicles with PGE2 at a concentration of 1 µg/ml and the broad inhibitor at a concentration of 5 µM and the specific inhibitor at a concentration of 50 µM.

A non-significant increase was detected in the stress at failure and energy of the groups treated with the specific MMP-3 inhibitor over the broad-spectrum MMP inhibitor (Figure 31 and 34). The stiffness of tissue cultured with the specific MMP-3 inhibitor showed a slight increase over the stiffness of those cultured with the broad-spectrum MMP inhibitor, but the change was not statistically significant (Figure 32). The strains at failure showed no significant difference between the groups (Figure 33).

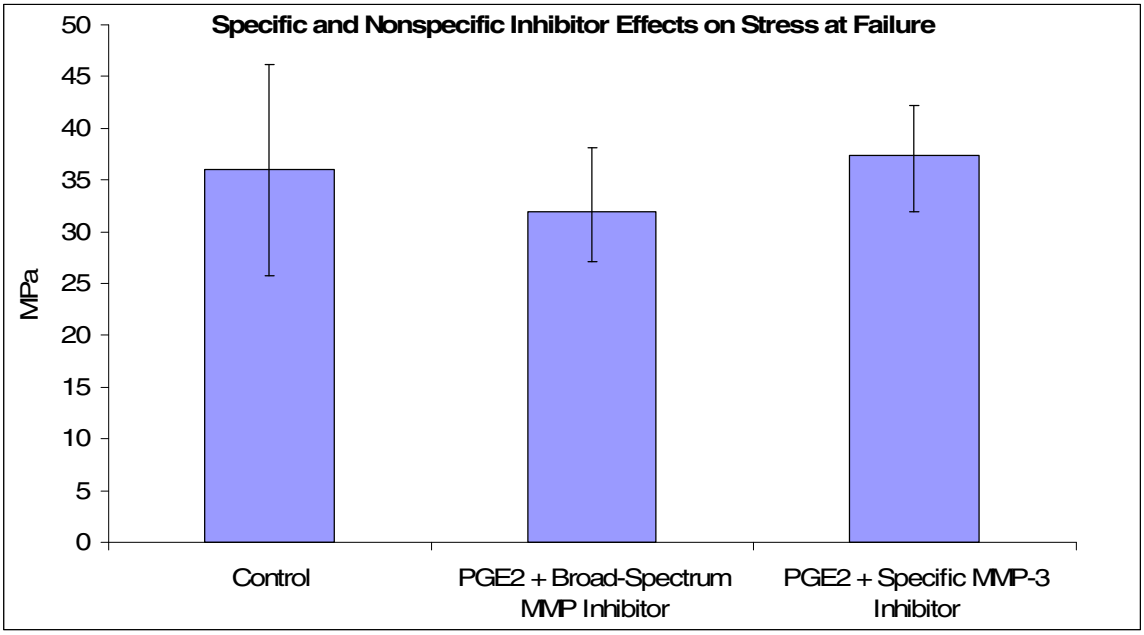

Figure 31: Maximum stress value reached during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor of any kind (Control), with PGE2 and the Broad MMP inhibitor (Ilomastat, 5 µM), or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

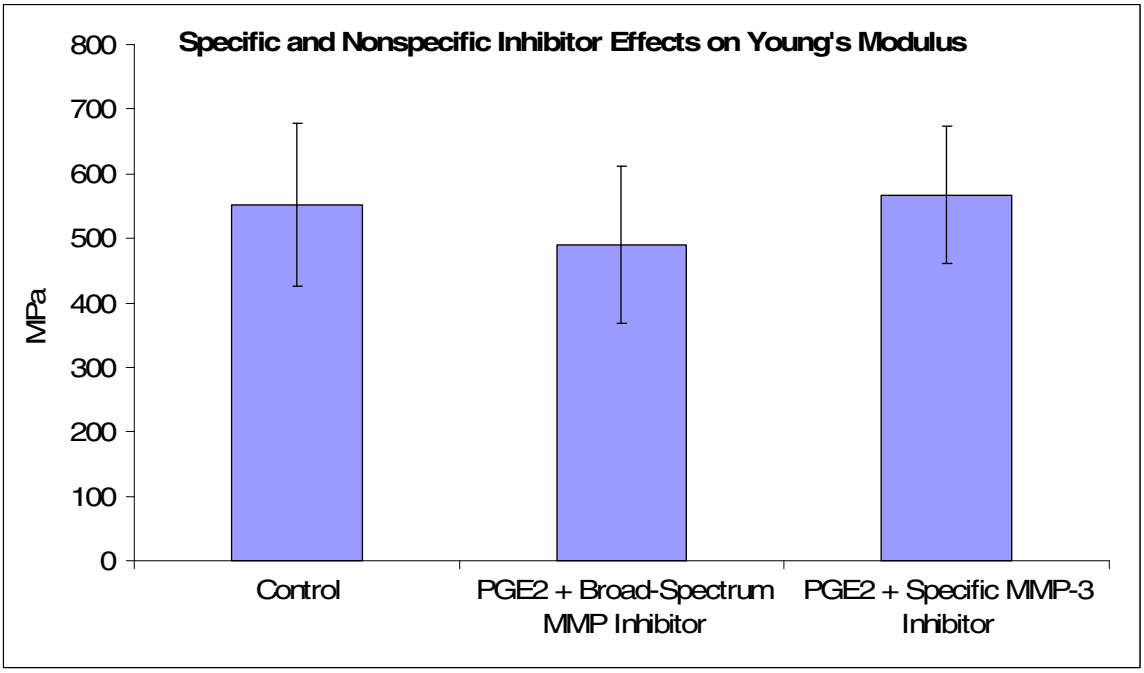

Figure 32: Young's modulus measured during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor of any kind (Control), with PGE2 and the Broad MMP inhibitor (Ilomastat, 5 µM), or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

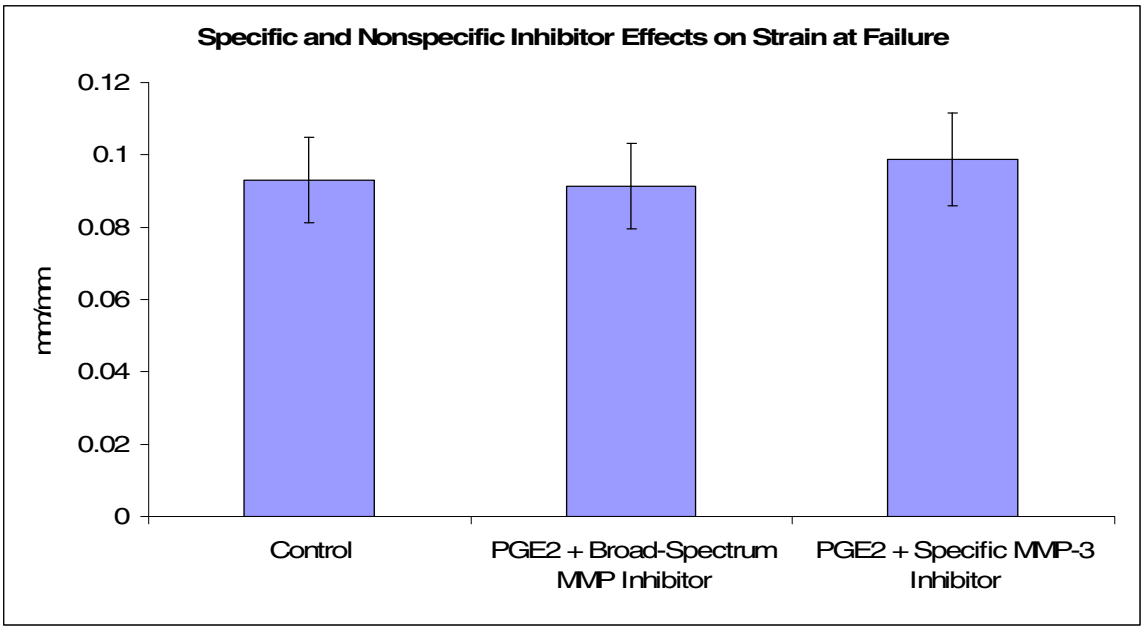

Figure 33: Strain measured during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor of any kind (Control), with PGE2 and the Broad MMP inhibitor (Ilomastat, 5 µM), or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

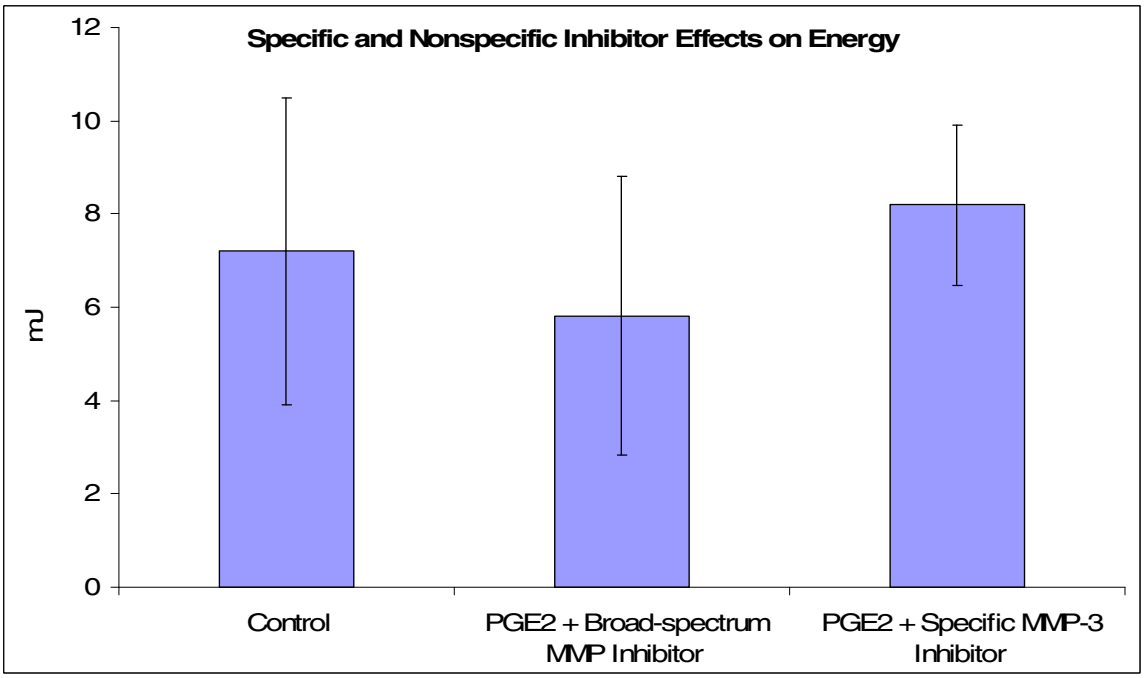

Figure 34: Energy measured during tensile failure test of rat tail tendon fascicles cultured without PGE2 (1µg/ml) or inhibitor of any kind (Control), with PGE2 and the Broad MMP inhibitor (Ilomastat, 5 µM), or with PGE2 and the specific MMP-3 inhibitor (50 µM) unloaded for 40 hours. Error bars indicate standard deviation. Sample sizes were as follows: Control (n=15), PGE2 (1µg/ml) plus broad MMP inhibitor (bMMPi, 5µM) (n=12), and PGE2 (1µg/ml) plus specific MMP-3 inhibitor (sMMP3i, 50 µM) (n=12).

Chapter 5

DISCUSSION

PGE2 has long been implicated as a precursor to tendinopathy development. During tendon loading there are increases in both PGE_2 levels and MMP levels in tendon [18, 35, 85, 86]. MMPs have been increasingly investigated for their role in tendinopathy development, yet the correlation between PGE2 levels and specific MMP synthesis and activity has only recently begun to be explored [118].

The cause of tendinopathy has been classically described as an overuse phenomenon, resulting from an accumulation of fiber 'micro-injuries'. The exact molecular mechanisms are unknown, but it has been suggested that the local strain loss due to these fiber failures initiates a catabolic response in the tenocytes associated with that fibril [24]. This may include an increase in metalloproteinase activity similar to the mechanisms seen in load-deprived tendon [37, 113, 119].

PGE2 has been shown to decrease collagen type I production as well as increase MMP-1 and 3 expression and synthesis in cultured tendon fibroblasts [73, 118]. The *in vitro* system used in our study has been developed to study the effects of PGE2 on rat tail tendon fascicles during unloaded and loaded culture. PGE2 was shown to decrease the mechanical properties of the tissue after 40 hours in unloaded culture, and to illicit no change in response after loaded culture. This is consistent with an increase in MMP, as it

has been shown that MMP expression is inhibited in statically loaded rat tail tendon *in vitro* [37] and collagenase is unable to cleave tendon in tension [119].

The strain measurements made during the loaded culture showed a significant contraction of the tendons treated with PGE2 after the initial 24 hour unloaded culture. It has been well documented that certain fibroblasts can assume a contractile phenotype *in vitro* which may account for this shortening [120]. However, it is interesting that PGE2 appears to be the cause of this contraction, as the process has also been shown to depend on increased MMP activity [121]. It has been shown previously that the Ilomastat MMP-inhibitor used in this study will limit contraction of a fibroblast-populated type I collagen lattice and rat tail tendon contraction in culture [121, 122]. This further suggests PGE2's actions are due to an increase in MMP activity in the culture system.

To help determine if MMP activity was responsible for the changes observed in the PGE2-treated tendon, we utilized a broad-spectrum MMP inhibitor. The inhibitor, Ilomastat, was chosen because it had been previously used in a rat tail tendon culture system to inhibit MMP-13, specifically, and is known to inhibit most MMPs [122, 123]. Ilomastat eliminated the decreases in material properties caused by exogenous PGE2 in the 40 hour unloaded culture system. These findings suggest that PGE2 acts to reduce material properties in the rat tail tendon after 40 hours of unloaded culture through an increase in MMP activity.

It has been shown that the cytokine IL-1β may act on tendon through an increase in PGE2 coupled with an increase in the EP4 receptor. This then amplifies the cellular response to PGE2, which results in increased expression of MMP-1 and MMP-3 [81].

Early increases in collagenase are thought to potentially contribute to tendinopathy development over time, but the role of MMP-3 is not well understood.

PGE_2 is applied clinically to induce cervical remodeling and softening seen during labor. As in tendon, PGE_2 in the cervix has been shown to effect cervical tensile strength and collagen organization via the EP4 receptor [82, 99]. However, the effect of PGE_2 on MMP-3 expression, synthesis and activity has been explored in more depth in the cervix. It has been found that MMP-3 expression parallels cervical tensile strength changes during pregnancy more closely than collagenase [77] MMP-3 is a potent activator of MMP-1, as well as many of the other MMPs, and its increased activity level in PGE2-treated tendon may be the instigator of degeneration. MMP-3's ability to cleave extracellular glycoproteins may also affect intrafibrillar interactions and alter tendon mechanical properties without cleaving collagen I [36]. In order to investigate the role of MMP-3 in tendon, we employed a specific inhibitor of MMP-3 in our tissue culture system.

The failure properties of rat tail tendon fascicles cultured with PGE_2 and a specific MMP-3 inhibitor did not display a significant difference, though there was a trend for the fascicles treated with the MMP-3 inhibitor to have increased mechanical properties. The PGE_2-treated tendon tissue showed less drastic decreases in properties in these groups than had been seen in our earlier work. We have proposed that these discrepancies may be due to differences between the PGE_2 itself or the different animals' sensitivity to its effects. The broad-spectrum inhibitor has been used in a rat tail model previously, but was only validated to reduce MMP-13 (equivalent to human MMP-1) in that model [122]. This data shows a slight indication that MMP-3 has the potential to be

an important mediator of tendon degeneration, as it maintained the mechanical properties better than any other treatment group after 40 hours of unloaded culture. The inability to detect significant changes in many of these properties may be due to the small sample size for these experiments.

There are many limitations involved in the experiments presented here, mainly stemming from the use of multiple rat populations. During our screening for rats, we attempted to normalize as many factors as possible in order to reduce variability. This included exclusively harvesting tails from female Sprague-Dawley retired breeder rats in the weight range of 300-400g. However, we did not limit the animals to a specific age range which has been shown to have significant effects on rat tail tendon mechanical properties [124]. Also, rats utilized for the PGE_2 time course experiments, the static loading experiments, and the broad-spectrum Ilomastat MMP inhibitor experiments utilized rats from a single population, whereas the specific MMP-3 inhibitor experiments were completed with rats from another population. This would have introduced genetic variability that may account for the differential effects of PGE_2 on these tissue mechanical properties.

In order to further decrease variability between fascicles, the tissue could be harvested from a specific tendon location in the rat tail, either dorsal or ventral. Tendons in the same tail region would experience similar loads in vivo, and would thus have less mechanical property variation. Likewise, ensuring a random distribution of tendons from any given animal amongst treatment groups would decrease variability. In the studies presented here, the fascicles were isolated from each animal and combined in sterile media in a Petri dish. This accounted for randomizing fascicles from different animal

sources and different tendon locations within the tails, but could be improved in future work.

To combat the issues seen in these studies with variable effects of PGE_2on tendon fascicles isolated from different rat populations, a dose response of fascicles to PGE_2 could be completed. This would determine an ideal PGE_2 concentration that would elicit a response in a broader assortment of rat populations.

Other limitations in these experiments are due to the lack of biochemical and histological analysis. In order to develop a more complete picture of PGE_2's effects in tendon, biochemical analysis of collagen and glycosaminoglycan levels could be determined. Moreover, in order to definitively determine enzyme activity in tendon due to PGE_2 specific MMP-1 and MMP-3 assays could be employed. Using histology could also allow for a more precise determination of extracellular matrix changes due to PGE_2 and MMP action.

In conclusion, PGE_2's degeneration of tendon mechanical properties appears to be carried out via the action of MMPs. However, the key to treating tendon degradation disorders may be in determining the specific MMP responsible for these changes. The role of MMP-3 in tendon should be explored further, as inhibition of this enzyme increases the stress to failure and energy in tendon significantly over inhibition of collagenase. Isolating the early contributors to tendinopathy development may better direct future pharmaceutical treatments, and further help preventative measures.

Other future work can include an investigation of different loading regimens. This work explored static tensile loading, however a more physiologic loading protocol would involve cyclic loading of the tendon. The static loading device presented here

could be modified to accommodate a change in loading regimen, with either the tendon hanging bar cyclically moved up and down, or the base housing the culture tubes may oscillate to strain the tendons.

The work presented here attempts to investigate the relationship between PGE_2, collagenase, and MMP-3. While there are several limitations in this research, it does provide a beginning look at MMPs and MMP inhibition with PGE_2 exposure in tendon. In the future, these studies can be used as an initial basis for development of more loading regimes and treatments in order to further increase the understanding of PGE_2's effects in tendons and tendinopathy.

Appendix 1: Static Loading Device Drawings

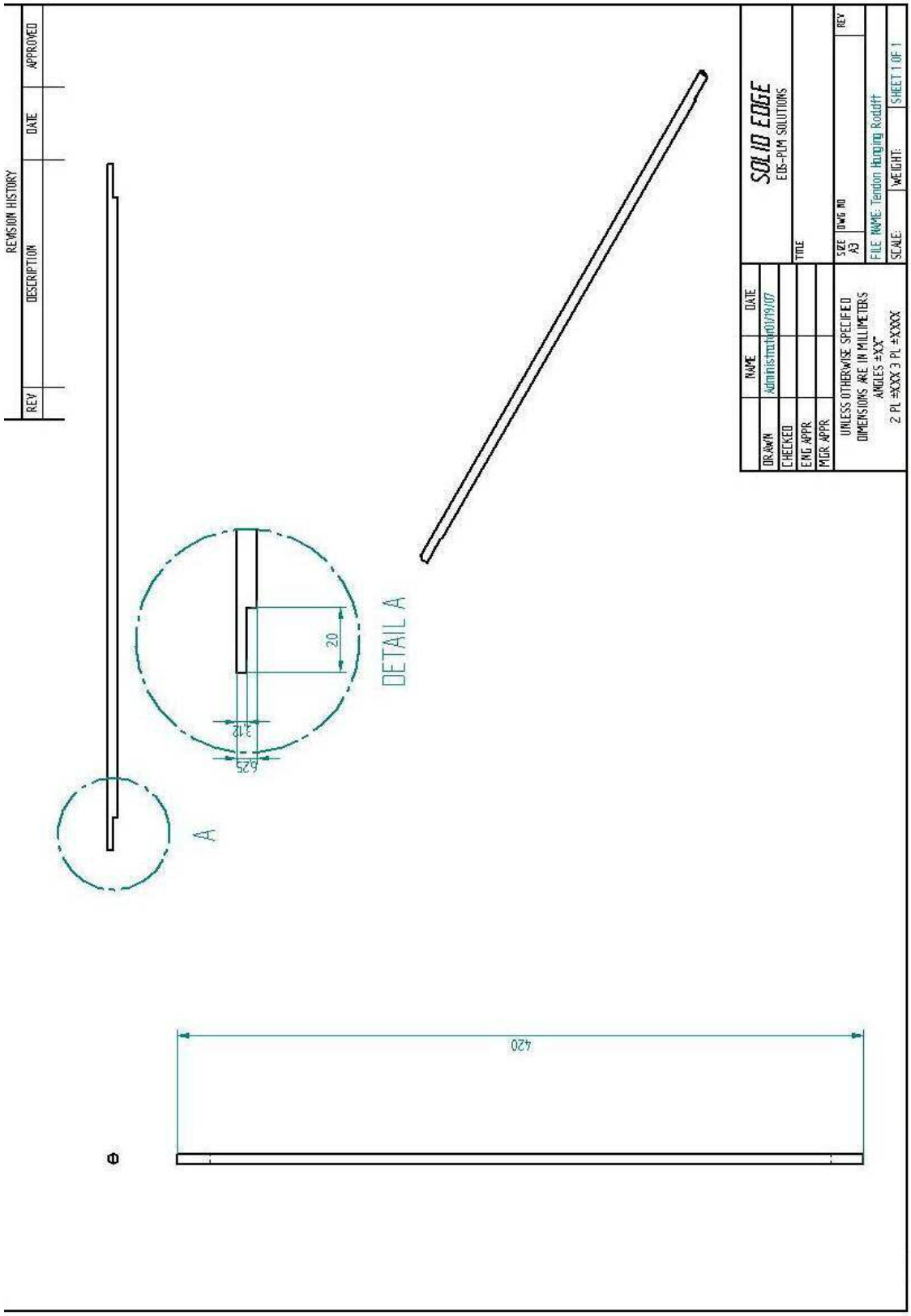

Figure 35: Tendon hanging rod for statically loading tendon in incubator

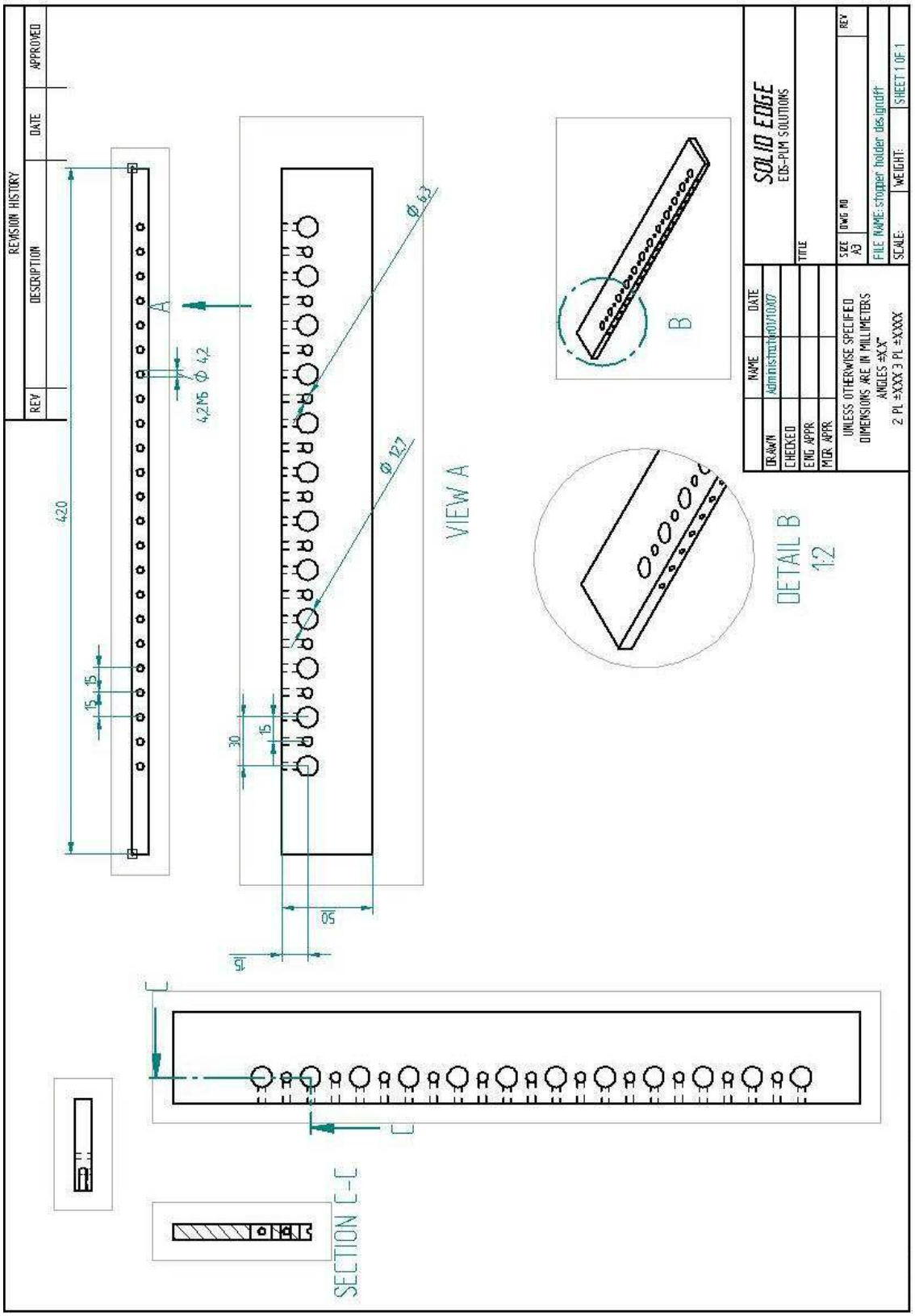

Figure 36: Stopper holder device for incubator

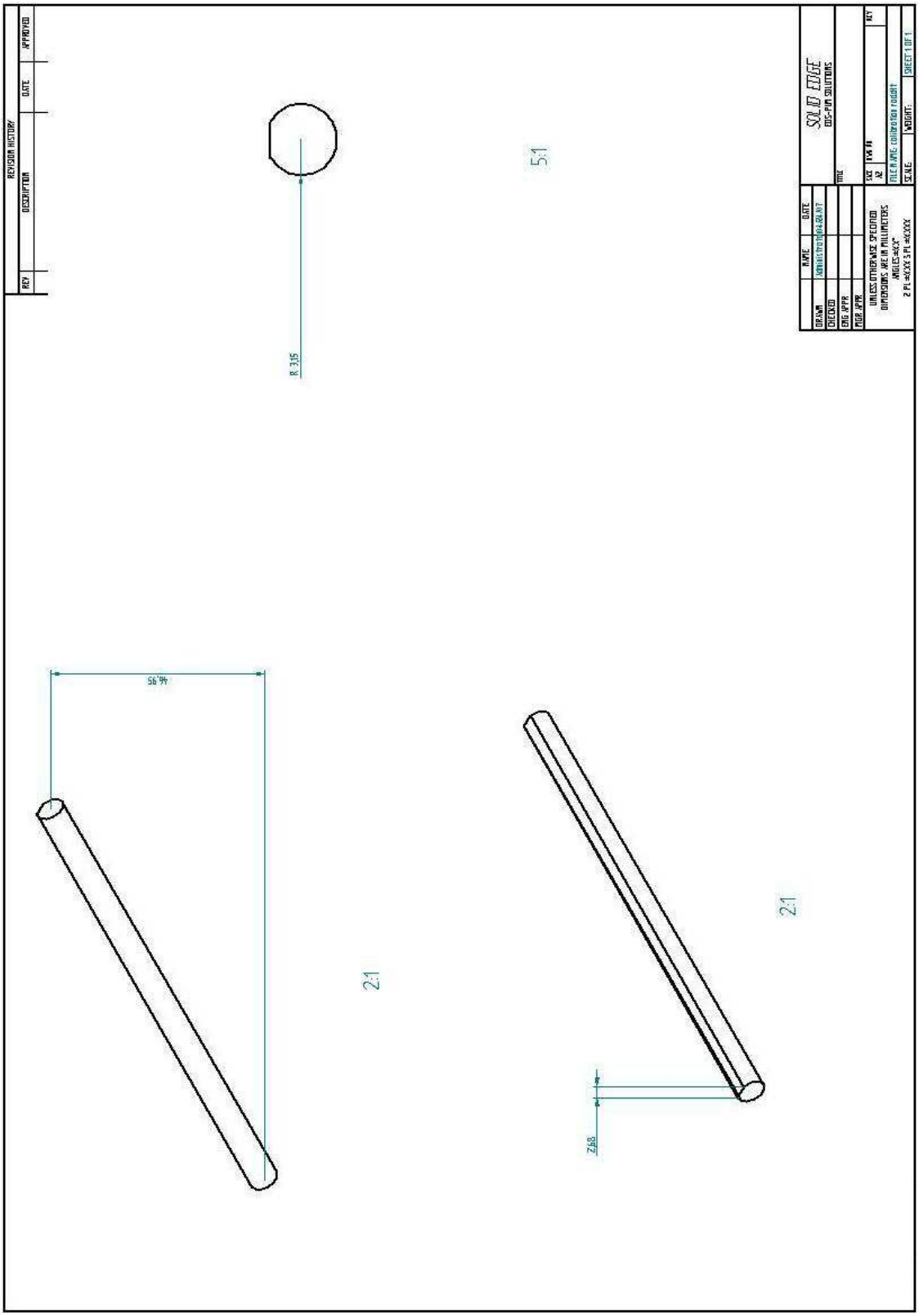

Figure 37: Calibration rods for visual strain analysis

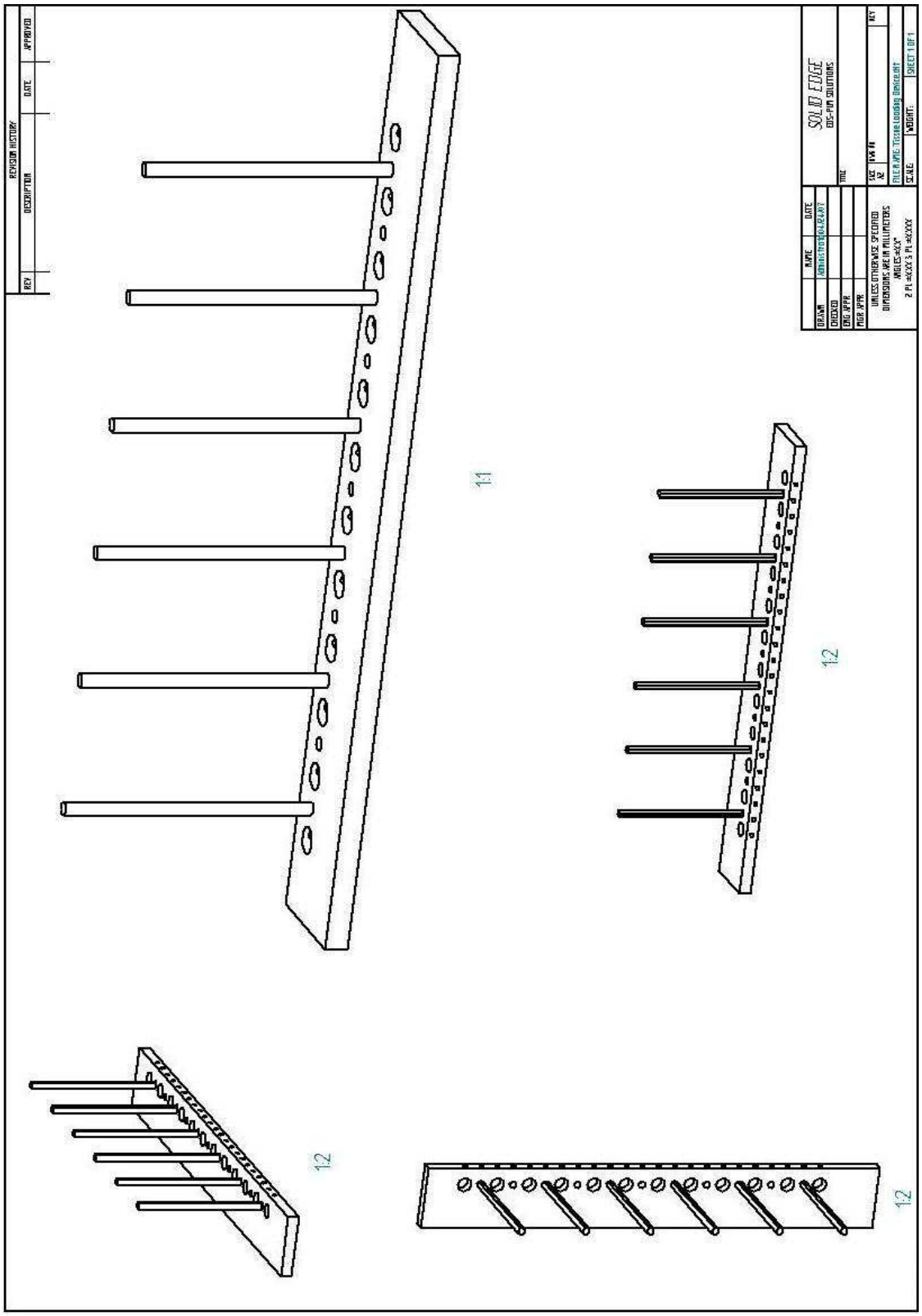

Figure 38: Stopper holder device assembled with calibration rods

Appendix 2: Prostaglandin E_2 Concentration Calculation

The PGE_2 concentration of 1 μg/ml used in the culture system was primarily based on PGE_2 levels seen previously in a cyclic loading tissue culture system in our laboratory. PGE_2 levels in culture medium were measured after 12 days of 4 hour per day cycles of a 12 MPa load. This system utilized chicken digital flexor tendons, which were found to produce approximately 70,000 pg/ml PGE_2 [125]. The vast difference in cross-sectional area between the digital flexor tendon and rat tail tendon (3 mm^2 for chicken tendon as compared with ~0.1 mm^2 for rat tail tendon), as well as the much higher media volume (37.5 ml media in chicken experiments compared with 1.5 ml media for rat tail experiments), were used to scale the concentration for the current system and ensure the final concentration is considered relatively stable [126]. PGE_2 was obtained from Cayman Chemicals (Dinoprostone, Catalog #14010) as 1 mg of solid. The calculations of amount per milliliter of media are outlined below.

Prostaglandin E_2; Dinoprostone (CAS 363-24-6)

Arrives: 1 mg Working Concentration: 1 μg/ml

Molecular Weight: 352.5

Reconstitute PGE_2 in 1 ml 100% ethanol: $[PGE_2]_{vial}$ = 1 mg/ml

Experimental Media Volume = 1.5 ml

<u>From Chicken tendon study:</u>

- 12 MPa Load-induced $[PGE_2]$ = 70,000 pg/ml
- Chicken tendon volume = ~120 μL

- 70,000 pg/ml PGE_2 x 37.5 ml media = 2.59 μg PGE_2

Rat tail tendon volume (assuming 300μm diameter and 70 mm length) = ~10μL

Scale PGE_2 amount: 2.59 μg PGE_2 (10μL rat tail/120 μL chicken) = 0.216 μg PGE_2

Want approximately 0.216 μg PGE_2 in 1.5 ml media: 0.144 μg/ml

Choose **1 μg/ml PGE_2**

$[PGE_2]_{vial}$ = 1000 μg/ml, need 1:1000 dilution

1 μL PGE_2 per 1 ml media

Appendix 3: Inhibitor Concentration Calculations

Appendix 3.1

Broad-spectrum MMP Inhibitor (Ilomastat, GM6001) Concentration

The broad-spectrum MMP inhibitor, Ilomastat, used in our rat tail tendon model was determined from previous studies in which it proved effective [122, 127]. The inhibitor was obtained from Chemicon International (GM6001 [Ilomastat] MMP Inhibitor, Catalog # CC1000). It arrived in a concentration of 1 mg/ml (2.5 mM) in DMSO. The calculations for determining the amount per milliliter of media outlined below.

GM6001 [Ilomastat] MMP Inhibitor

Arrives: 1 mg/ml = 2.5 mM Working Concentration: 5 μM

$2.5\ mM = 2.5 \times 10^3\ \mu M$

$2500\ \mu M / 5\ \mu M = 500\ X$

(Must be diluted by a factor of 500)

2 μL stock ilomastat per 1 ml media = [Ilomastat] of 5 μM

Appendix 3.2

Specific MMP-3 Inhibitor Concentration

The chemical name for the specific MMP-3 inhibitor is 4-(4'-Biphenyl)-4-hydroxyimino-butyric acid. It was obtained from Calbiochem (MMP-3 Inhibitor VI, Catalog #444265) as 5 mg of a white solid. The concentration used in our culture system was determined from the K_i values found both *in vivo* and *in vitro* [128, 129]. We determined that a 50 μM concentration of this inhibitor would be effective to block MMP-3 activity. The calculations for determining the amount per milliliter of media outlined below.

MMP-3 Inhibitor VI

Arrives: 5 mg

Working Concentration: 50 μM

Molecular Weight: 269.3

Solubility: DMSO

Determine moles of inhibitor: 0.005g / 269.3 = 18.56 μmol

Reconstitute with 1 ml DMSO: Inhibitor concentration = 0.0186 M ~ 0.02 M

0.02 M / 50 μM = 400 X

(Must be diluted by a factor of 400)

1 μL stock MMP-3 inhibitor per 400 μL media = [MMP-3 Inhibitor] of 50 μM

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